Host cell factors influencing intracellular survival and replication of *Legionella pneumophila*

Dissertation

zur Erlangung des akademischen Grades

doctor rerum naturalium
(Dr. rer. nat.)

im Fach Biologie
eingereicht an der Mathematisch-Naturwissenschaftlichen Fakultät I
Humboldt-Universität zu Berlin

von
Dipl.-Biol. Cecilia Maria Amelie Engels
* 13.07.1979 in Konstanz

Präsident der Humboldt-Universität zu Berlin
Prof. Dr. Dr. h.c. Christoph Markschies
Dekan der Mathematisch-Naturwissenschaftlichen Fakultät I
Prof. Dr. Lutz-Helmut Schön

Gutachter/innen:

1.) Prof. Dr. A. Flieger

2.) PD Dr. K. Heuner

3.) Prof. Dr. T. F. Meyer

Tag der mündlichen Prüfung: 15.09.2009

“But like of each thing that in season grows”

Love´s Labour´s Lost

Index

Index 3
Abstract 6
1 Introduction 9
1. 1 *L. pneumophila* – an aquatic microbe goes pathogen 9
1. 2 *L. pneumophila* - infection of protozoa 10
1. 3 *L. pneumophila* - infection of professional phagocytes 11
Adhesion, Phagocytosis 11
Inhibition of phagosome-lysosome fusion 12
Establishment of the *Legionella* containing vacuole (LCV) 13
Modulation of host signal transduction mechanisms 15
Replicative phase 16
Evasion from the host cell 16
1. 4 Cellular trafficking in *L. pneumophila* infection 17
Rab GTPases 20
SNARES 22
Golgi apparatus 25
1. 5 Protein phosphorylation as a method of molecular control 26
1. 5. 1 Intracellular communication via tyrosine phosphorylation upon *L. pneumophila* infection 28
1. 6 Anti-apoptotic NF-κB-signalling in *L. pneumophila* infection 29
1. 6. 1 NF-κB 29
1. 6. 2 Biphasic NF-κB activation upon *L. pneumophila* challenge 31
1. 6. 3 *L. pneumophila* virulence traits in NF-κB activation 32
1. 6. 4 Specific anti-apoptotic effectors of *L. pneumophila*: SidF, SdhA and SdbA 33
2 Material and Methods 34
Material 34
2. 1 Equipment 34
2. 2 Material 34
2. 3 Software 35
2. 4 Organisms 35
2. 4. 1 Cell culture 35
2. 4. 1. 1 Media for cell culture 35
2. 4. 1. 2 Supplements for cell culture 36
2. 4. 2 Bacteria 36
2. 4. 2. 1 Antibiotics 36
2. 5 Chemical reagents 36

2. 6 Antibodies 37
2. 7 siRNA 38
2. 8 Kits 39
Methods 39
2. 9 Eukaryotic cells 39
2. 9. 1 Cultivation of human epithelial cells 39
2. 9. 2 Cryo-conservation of eukaryotic cells 40
2. 9. 3 Defreeze and re-cultivation of eukaryotic cells 40
2. 9. 4 Amoeba cultivation 40
2. 10 Bacteria 41
2. 10. 1 Growth of *L. pneumophila* 41
2. 10. 1. 1 *Legionella* preparation for infection 42
2. 11 *L. pneumophila* infection of eukaryotic cells 42
2. 11. 1 Gentamicin-protection-Assay 42
2. 11. 2 *L. pneumophila* infection of amoeba 42
2. 12 Nucleic acid methods 43
2. 12. 1 siRNA transfection of eukaryotic cells 43
2. 13 Protein biochemical methods 44
2. 13. 1 Detection of protein amount (BCA) 44
2. 13. 2 Discontinuous SDS polyacrylamide gel electrophoresis (SDS-PAGE) 44
2. 13. 3 Immunoblot (Western blot) 46
2. 13. 4 Detection of antigen-antibody reaction 47
2. 13. 5 Chemo luminescence (ECL) detection 47
2. 13. 6 Membrane stripping 48
2. 13. 7 Protein co-immuno-precipitation (P-Tyr Kit) 48
2. 13. 8 Molecular mass determinations by MALDI mass spectrometry 49
2. 14 Fluorescence based methods and microscopic studies 49
2. 14. 1 Immunofluorescence staining 49
2. 14. 2 Confocal microscopy 50
2. 14. 3 NF-κB activation readout-system 50
3 Results 52
3. 1 Establishment of optimal *L. pneumophila* infection model setting 52
3. 1. 1 Growth of different *L. pneumophila* wild type strains in HeLa model cells 52
3. 1. 2 Growth of *L. pneumophila* Philadelphia01 wt at MOIs 10, 100 in HeLa model cells 54
3. 1. 3 Growth of *L. pneumophila* JR32 Δ*dotA* for 24 h in HeLa model cells 55
3. 1. 4 Summary 56
3. 2 “Focussed mini-screen” set up 57

3. 2. 1 Transfection of siRNA in HeLa model cells 57
3. 2. 1. 1 Protein amount validation after siRNA knock-down (time course) 57
3. 2. 1. 2 Protein amount validation after siRNA knock-down (different siRNA sequences) .. 58
3. 2. 2 Target proteins 59
3. 2. 3 “Focussed mini-screen” 63
3. 2. 4 *L. pneumophila* replication (model graphs) 65
3. 2. 5 Fold of replication 69
3. 2. 6 Putative interaction-site of *L. pneumophila* effector LidA with the SNARE-complex ... 70
3. 2. 7 Localisation of specific ER- & Golgi apparatus-proteins in *L. pneumophila* infection .. 72
3. 2. 8 Summary 75
3. 3 Protein-phosphorylation detection and characterisation 76
3. 3. 1 Phospho-Tyrosine detection in *L. pneumophila* infected *Acanthamoeba castellanii* .. 76
3. 3. 2 Purification & identification of the detected phospho-tyrosine containing protein 77
3. 3. 3 Summary 80
3. 4 NF-κB activation and detection after *L. pneumophila* infection 80
Biphasic NF-κB activation after *L. pneumophila* infection 81
3. 4. 1 Growth of *L. pneumophila* Corby wt and Phil01 wt in HeLa and A549 model cells 83
3. 4. 2 *L. pneumophila* mutants κ*sdbA* and κ*sdhA* growth in comparison 84
3. 4. 3 Summary 86
4 Discussion 87
4. 1 *L. pneumophila*-infection model 87
4. 2 *L. pneumophila* hijacks its host trafficking pathway & the role of Golgi apparatus structure 88
The role of Golgi apparatus and its structure 92
4. 3 Protein phosphrylation on host protins upon *L.pneumophila* infection 94
4. 4 NF-κB response upon *L. pneumophila* infection 96
Conclusion 99
Abbreviations 100
Acknowledgements 102
Publications 103
Literature 105

Abstract

Legionella pneumophila is the causative agent of Legionnaires´ disease. The bacterium's pathogenicity is based on its ability to survive and multiply efficiently inside human alveolar cells. Therefore, *L. pneumophila* is not only an important pathogen, but can also be used as a probe to investigate host cell function as for example, in the cellular trafficking pathway. In this study, we establish a new model of how this pathogen efficiently constructs its replicative niche, the *Legionella* containing vacuole (LCV), inside the host cytosol, enabling its dissemination. To investigate the mechanisms that lead to effective exploitation of the host cell, we down-regulated specific host cellular proteins via siRNA technology and measured the subsequent impact on *L. pneumophila* replication. The results suggest that the LCV mimicks the Golgi apparatus and via this mechanism hijacks host cellular vesicular trafficking. The *L. pneumophila* secreted effector protein LidA, located within the LCV, is shown to have a SNARE-like motif, suggesting a SNARE like sole connected to the LCV.

Since it is known that cellular signalling proteins are controlled via phosphorylation and dephosphorylation, we went on to search for specifically modulated host cell proteins after *L. pneumophila* infection. The cross-talk of the pathogen with its host via phosphorylation has been connected to several sub-cellular activities leading to, for instance, cytoskeleton rearrangement and signalling events including anti-apoptosis pathways. Here we used a phosphorylated tyrosine antibody resulting in the detection of an amoeba serine-threonine-kinase, phosphorylated at its tyrosine residue. This kinase shows homologies to the human GSK3 of the wnt-signalling pathway. ("Wnt" is merged from the names of the homologues genes Wg (*Drosophila melanogaster*) and Int (mouse) both employed in evolutionary developement.)

The final part of this work concentrated on anti-apoptotic signalling events induced upon *L. pneumophila* infection. It is known that during *L. pneumophila* infection the activation of NF-κB and subsequent translocation of p65 from the cytosol into the nucleus follows a biphasic pattern. One short peak of activation is induced upon contact with bacterial flagellin, succeeded by a permanent Dot/ Icm type IV secretion system-dependent activation. In this study, we found the *L. pneumophila* mutant lacking the Dot/ Icm effector SdbA to be unable to activate NF-κB. This mutant also showed impaired growth in epithelial cells. This is remarkable due to the high redundancy of the *L. pneumophila* effector system, meaning deletion of a single effector rarely has such a big impact on replication.

Taken together this work demonstrates, the manifold ways in which *L. pneumophila* on the one hand side establishes its niche to ensure replication and on the other hand side to bars its host cell from suicide. All of this is managed by mimicking cellular processes.

Zusammenfassung

Legionella pneumophila ist der Erreger der Legionärskrankheit. Die Pathogenität des Bakteriums basiert auf seiner Fähigkeit innerhalb menschlicher Lungenzellen zu überleben und sich zu vermehren. Demzufolge ist *L. pneumophila* nicht nur interessant als wichtiges Pathogen, sondern kann auch als Sonde verwendet werden, um allgemeine intrazelluläre Ereignisse zu untersuchen. Ein Beispiel hierfür ist die, durch das Pathogen gestörte, intrazelluläre Kommunikation zwischen den Organellen des endoplasmatischen Retikulums (ER) und dem Golgi Apparat (GA). In der vorliegenden Studie schlagen wir ein neues Modell vor, wie das Bakterium erfolgreich seine replikative Nische, die *Legionella* Vakuole (LV), innerhalb des Zytosols aufbauen könnte, um seine Ausbreitung zu garantieren. Um die Mechanismen für die erfolgreiche Ausbeutung der Wirtszelle gezielt untersuchen zu können, haben wir mit Hilfe von siRNA spezifisch verschiedene Wirtszellproteinen herunterreguliert und den Einfuß der Abwesenheit dieser Proteine auf die Vermehrung von *L. pneumophila* gemessen. Die Ergebnisse wiesen darauf hin, dass die LV möglicherweise den Golgi Apparat imitiert und auf diese Weise den zellulären Vesikeltransport umleitet. Diese Theorie wurde durch *in silico* Ergebnisse unterstützt, die in der Proteinsequenz des *Legionella* Effektor-Proteins LidA, das auf der Vakuole lokalisiert ist, ein SNARE-ähnliches Motiv zeigte. Dies weist auf ein auf der Vakuole lokalisiertes SNARE-Erkennungsmotiv hin, das notwendig sein könnte, um zelluläre Transportvesikel zu koppeln.

Aus dem Wissen heraus, dass *L. pneumophila* in der Lage ist, die Aktivierung der zellulären Proteine Arf1 und Rab1 durch Phosphorylierung und Dephosphorylierung zu regulieren, machten wir uns auf die Suche nach Proteinen, die auf Infektion hin modifiziert werden. Die Kommunikation von Wirt und Pathogen über Phosphorylierung ist bekannt im Bezug auf pathogenspezifische Modifikation des Zytoskeletts und Signalkaskaden in der Anti-Apoptose. Für diese Studie wurde ein Antikörper verwendet, der spezifisch phosphorylierte Tyrosinreste erkennt. Dies resultierte in der Detektion einer Serin-Threonin-Kinase in der Amöbe *Acanthamöba castellanii*, die an einem Tyrosinrest phosphoryliert ist. Diese Amöben-Kinase wies *in silico* Homologie zu der humanen GS-Kinase 3 des Wnt-Signalwegs, bekannt aus der Forschung der embronalen Entwicklung bei Drosophila, auf.

Der letzte Teil dieser Arbeit konzentrierte sich auf die, durch eine *L. pneumophila*-Infektion ausgelöste, anti-apoptotische Signalkaskade. Es ist bekannt, dass auf eine Infektion hin NF-κB aktiviert wird. Dies führt dazu, dass p65 in den Zellkern wandert und dort als Transkriptionsfaktor aktiv wird. Diese Translokation geschieht in 2 zeitversetzten Phasen. Eine Aktivierungsspitze wird nach dem Kontakt mir bakteriellem Flagellin gemessen, gefolgt, von einer dauerhaften Aktivierung, abhängig von einem funktionierenden Dot/ Icm Typ-IV-Translokationssystem. In dieser Arbeit stießen wir auf eine *L. pneumophila* Mutante, die den Dot/ Icm-Effektor SdbA nicht bildet, und die daraufhin NF-κB nicht aktivieren kann. Diese Mutante war ebenfalls nicht in der Lage, sich in Epithelzellen zu vermehren. Dies ist außergewöhnlich, da das *L. pneumophila* Effektor Repertoire so redundant ist, dass die Abwesenheit eines einzigen Effektors selten einen so starken Einfluss auf die Replikation hat.

All diese Ergebnisse zeigen zusammengenommen, auf wie vielen verschiedenen Ebenen *L. pneumophila* in der Lage ist, seine Wirtszelle zu manipulieren, um einerseits die nötige Nische für

seine Vermehrung zu etablieren und andererseits die Zelle am Selbstmord zu hindern. Dies geschieht durch Imitation zellulärer Prozesse.

1 Introduction

The bacterial human pathogen *Legionella pneumophila* - one of the more recently discovered pathogens by McDade (McDade *et al.* 1977) - infects the lung via aerosols and causes severe pneumonia. *L. pneumophila´s* pathogenicity is primarily based on its ability to replicate within the cellular lumen of alveolar macrophages. In the non-human environment the pathogen lives and replicates within its natural protozoan hosts. This dual host system renders *L. pneumophila* an especially interesting model organism: a ubiquitous environmental germ as well as a pathogenic agent. In order to highlight its singular characteristics within the community of infectious agents we shall focus on *L. pneumophila,* but shall - from time to time - revert to the characteristics of other intracellular pathogens to highlight *L. pneumophila.*

1. 1 *L. pneumophila* – an aquatic microbe goes pathogen

L. pneumophila is naturally found in fresh water. It is a rod shaped Gram-negative bacterium with a unipolar flagellum. *L. pneumophila's* natural habitat is the aquatic association with protozoa e.g. *Acanthamoeba*, *Naegleria*, *Hartmanella* und *Tetrahymena* (Brand & Hacker 1997). They also survive planctonically in biofilms (Steinert *et al.* 2002) as for example in air-conditioning, whirlpool and shower systems where the pathogen finds optimal life conditions. Via these technical vectors the agent is able to translocate through aerosols via microaspiration into the human lung where it causes legionellosis, an atypical pneumonia. This is why Chen *et al.* in 2004 termed *L. pneumophila* "an accidental human pathogen". Its ability to replicate within human cells protects the bacterium from humoral or cellular agents of the immune system. To note, for *L. pneumophila*: Human to human transfer has not yet been observed.

Taxonomically, *L. pneumophila* are classified into more than 42 species and 65 serogroups (Lück & Helbig 1997). Out of the 42 species at least 16 are classed human pathogen. Besides *L. pneumophila* serogroup 1, identified as the cause in ca. 90 % of manifested legionellosis. Also *L. micdadei*, *L. bozemanii* and *L. longbeachae* are found to most potently cause this atypical pneumonia (Atlas *et al*. 1999).

To subsist in fresh water reservoirs, *L. pneumophila* alternates between an intracellular replicative form and an extracellular transmissive form, a cellular differentiation governed by nutrient supply (Swanson & Hammer 1999). *L. pneumophila´s* ability to replicate within professional phagocytes is central to establishing infection. Besides replicating in alveolar macrophages, blood monocytes and polymorph nucleic leukocytes *L. pneumophila* also replicates within non-professional phagocytic cells as for example epithelial cells type I and type II (Abu Kwaik 2000) as shall be described later.

From the epidemiologic point of view *L. pneumophila* propagated in aerosols causes either *Legionella*-pneumonia (Legionnaires´ disease) or a self-limiting respiratory infection, the so-called Pontiac fever. In the U.S. each year estimated 8,000-18,000 cases are hospitalised. However, accurate data reflecting the true incidence of disease are not available due to under-utilization of diagnostic testing and under-reporting. The majority of reported cases are sporadic. Therefore the incidence of *L. pneumophila* infection should be considered higher than reported. Travel-associated

outbreaks, outbreaks in community settings, and nosocomial and occupational outbreaks – in addition - are common (Centres of Disease Control and Prevention 2009). In Germany the Robert-Koch-Institute reports 400 - 600 cases a year but these numbers are also believed to misjudged the real numbers. Rough estimations reach up to 30,000 cases a year world wide. The disease manifests in acute fever (> 40°C) after an incubation period of 2-10 days accompanied by cough, chill, headache and, ultimately, pneumonia (Lück & Helbig 1997, Susa *et al*. 1997). Untreated legionellosis is lethal in 10-25 % of the cases – dependent on the patient's immune status. *L. pneumophila* are opportunistic bacteria depending for virulence on a lowered immune status. Therapy is based on membrane collocating antibiotics as for example erythromycin, rifampicin and ciprofloxacin (Brand & Hacker 1997).

1. 2 *L. pneumophila* - infection of protozoa

The intracellular replication of *L. pneumophila* in protozoa (Fig 1. 1) is crucial to its subsistence in the environment and - in addition - safeguards it effectively from decontamination measures. Plus the bacterium is endowed with another practical survival trait: *L. pneumophila* conditioned in a VBNC (viable but not culturable) state may be reactivated while passing through amoeba and regain infectivity for human cells (Fields 1996, Steinert *et al*. 1997). Ultra-structure analysis via electron microscopy showed that the *L. pneumophila* life-cycle inside human phagocytic cells resembles the one in protozoa (Abu Kwaik 1996, Gao *et al.* 1997, Harb *et al.* 1998).

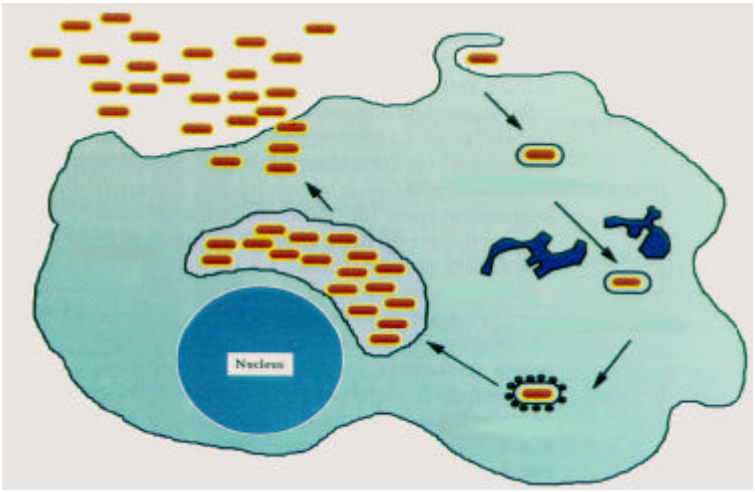

Fig. 1. 1. Life-cycle of *L. pneumophila* in a phagocytic cell.
L. pneumophila enters the eukaryotic cell via phagocytosis („coiling" or conventional), and later on avoids phagosome-acidification and „oxidative burst". For vacuole construction it associates with rough ER and starts intracellular replication within the replicative vacuole. After completed replication, the host-cell lyses and re-infection of new cells is possible (modified after Roy *et al.* 1999).

The entry mechanism of *L. pneumophila* into protozoa differs distinctly from its absorption into human cells (King 1991, Abu Kwaik *et al*. 1994). Contrary to macrophages, where phagocytosis is easily inhibited by cytochalasin D - protozoa do not seem to employ microfilament or microtubule dependent phagocytosis (King *et al.* 1991, Harb *et al.* 1998). The initial interaction between parasite and host-cell is adhesive, via bacterial ligands and receptors on the host-cell surface. *L. pneumophila´s* adsorption into the protozoan-host *Hartmannella vermiformis* is due to a special receptor, identified as a lectin that can be inhibited using galactose/ N-acetylgalactosamin (Gal/ GalNAc). During *L. pneumophila*-host interaction (ligand-connection) this lectin is dephosphorylated at a tyrosyl site and is homologous to the β-integrin-similar 170 kDa protein of the pathogen protozoon *Entamoeba histolytica*

(Venkataraman *et al.* 1997). This phosphorylation leads to multiple cytoskeletal rearrangements inside the host cell (Finlay & Cossart 1997, Abu Kwaik 1998a). In addition the invasion of *L. pneumophila* into protozoa triggers expression respectively repression of different host proteins (Abu Kwaik *et al*. 1994, Abu Kwaik & Pederson 1996, Abu Kwaik 1998d). Inhibition studies with cycloheximide showed that protein-biosynthesis is necessary for receptor-mediated endocytosis in *Hartmanella vermiformis* but not in *Acanthamoeba polyphaga* (Abu Kwaik *et al*. 1994, Harb *et al.* 1998). Neither galactose nor N-acetylgalactosamine or dephosphorylation of the 170 kDa lectin could inhibit phagocytosis of *L. pneumophila* into *Acanthamoeba polyphaga* (Harb *et al.* 1998). This heterogeneity of its entry-mechanisms indicates the amazingly adroit adaptation of *L. pneumophila* to different protozoan hosts. Thus it has been suggested that free-living amoebae may serve as a significant reservoir for pathogens in the environment, perhaps even as a "training environment" for the selection of virulence-related traits in these pathogens (Harb *et al.* 2000). The similarity between *L. pneumophila* infection of macrophages and protozoa suggests that in fact the bacterium's adaptation to the intracellular environment of protozoa provides the basis for its successful intracellular replication within human alveolar macrophages. Consequently in due course, *L. pneumophila* evolved into a human pathogen (Abu Kwaik 1996, Gao *et al.* 1997).

1. 3 *L. pneumophila* - infection of professional phagocytes

Microbial pathogens survive and kill bactericidal phagocytes or invade host cells and proliferate within the cell in niches protected from humoral and cellular components of the immune system (Nagai & Roy 2003, Rosenberger & Finlay 2003, Cossart & Sansonetti 2004). It is interesting to note that *L. pneumophila* falls into a class with other pathogens also using intracellular replication (Abu Kwaik 1996). *Chlamydia psittaci*, *Mycobacterium tuberculosis*, *Salmonella typhimurium* and *Toxoplasma gondii* (Finlay & Falkow 1997, Holden *et al.* 2006) also employ the strategy of averting phagosome-lysosome fusion. *Listeria monocytogenes*, *Shigella flexneri, Rickttsia* or even the eucaryotic parasite *Trypanosoma cruzi* demonstrate distinctively different replication: they replicate by escaping from the vacuole and survive within the host-cell cytoplasm (Finlay & Falkow 1997). The obligate intracellular agent of the Q-fever *Coxiella burnetii* even replicates within the acidified host-cell phago-lysosome (Baca & Paretsky 1983).

In the case of *L. pneumophila*, its virulence enables the pathogen to adhere to its host cell, to be taken up by phagocytosis, to evade phagosome-lysosome fusion and to establish a *Legionella*-containing vacuole (LCV) by modulating host signalling transduction mechanisms and recruiting vesicles from the endoplasmic reticulum (ER). While inhibiting apoptosis of its host the bacterium replicates inside the cell and evades after disruption of the cell. How this is admistered in detail and which virulence factors are involved shall be the main topic of this chapter on *L. pneumophila's* life cycle in the human cell (Fig. 1. 2).

Adhesion, Phagocytosis

Extracellular virulence is primarily determined by surface proteins (Dowling *et al.* 1992). *L. pneumophila* has LPS-structural particularities for Gram-negative bacteria within the core and lipid-A

array. These account for the low pyrogenity and the especially high hydrophobicity of the *L. pneumophila* LPS. The latter might be of importance during the aerogene transmission (Zähringer *et al.* 1995, Lück & Helbig 1997). The role of *L. pneumophila* LPS during target cell adhesion has not been satisfactorily elucidated, although genes involved in LPS-synthesis could be identified (Zou *et al.* 1999, Lüneberg *et al.* 2000). Another surface resident virulence trait of *L. pneumophila* is given by its expression of a unipolar flagellum predominantly composed of one ca. 48 kDa subunit encoded by the *flaA*-gene (Heuner *et al*. 1995). Expression of this flagellum is triggered by the alternative σ^{28} factor and does not seem to be essential for the intracellular replication in *A. castellanii* (Heuner *et al.* 1997, Heuner 1997), though invasion of Δ*flaA* mutants in *A. castellanii* and in HL60 cells was reduced (Dietrich *et al.* 2000). Expression of the flagellum starts at the passage from logarithmic to stationary growth-phase and can affect – after host cell lysis – detection of new host organisms via motility expression (Byrne & Swanson 1998, Heuner *et al.* 1999, Dietrich *et al.* 2000).

Entry of *L. pneumophila* into macrophages or monocytes occurs either via the so-called "coiling phagocytosis" or via conventional phagocytosis (Horwitz 1984). Crucial for absorption into macrophages is the initial binding of *L. pneumophila* MOMP („major outer membrane protein") to the eukaryotic complement factors C3b and C3bi. The MOMP-C3b/ C3bi-complex then binds to the complementary receptors CR1 and CR3 on the macrophage surface. This leads to microfilament dependent phagocytosis (Payne & Horwitz 1987, Bellinger-Kawahara & Horwitz 1990, King *et al.* 1991). Alternatively opsonised *L. pneumophila* can also be internalised via Fc-receptors in a complement-dependent manner (Husmann 1992, Stone & Abu Kwaik 1998).

The heat shock protein Hsp60 – encoded by the gene *htpB* – belongs to the family for GroEL-chaperones and is estimated to have lipochaperon-function. Its exposed location to the surface stabilizes the membrane and has been found to influence adherence and invasion into HeLa-cells (Garduño *et al.* 1998b, Hoffman & Garduño 1999). In Legionellosis Hsp60 is an effective antigen. It is secreted in freshly generated phagosomes derived from monocytes (Weeratna *et al*. 1994, Fernandez *et al.* 1996, Garduño *et al.* 1998a, b). Virulent and avirulent *L. pneumophila* strains differ in their Hsp60 localisation in the outer membrane.

Important virulence factors translocated via secretion systems are the phospholipases. Phospholipases are responsible for destruction of membrane-associated phospholipids. It has been shown that several *L. pneumophila* species secrete phospholipase A. Phospholipase A specifically demolishes phospholipids of the alveolar- and lung surface („surfactant") culminating in the disintegration of the cell (Flieger *et al.* 2000a, b).

Inhibition of phagosome-lysosome fusion

Upon phagocytosis the bacteria live and replicate in a modelled membrane enclosed phagosome (Horwitz & Maxfield 1984). Thus the pathogen circumvents lysosomal fusion and reduces the danger of phagosomal acidification as well as the so called "oxidative burst" by the formation of bacteriocidic oxygen radicals in macrophages (Horwitz 1983, Horwitz & Maxfield 1984, Fields 1996, Roy 1999). Clemens and Horwitz observed a modulated composition of the phagosomal membrane and its containing of MHC I- and MHC II-molecules (major histocompatibility complex) during or after

phagocytosis (Clemens & Horwitz 1992, 1993). How the pathogen modulates its phagosome is an important factor for survival and is accomplished by a diverse set of virulence factors.

Establishment of the *Legionella* containing vacuole (LCV)

By 5 min post infection, the vacuole containing wild-type *L. pneumophila* avoids interactions with the default endocytic/ lysosomal pathway. By 15 min post infection, the limiting vacuolar membrane is decorated with smooth vesicles (Swanson & Isberg 1995, Bozue & Johnson 1996). For the maturation of the nascent *Legionella* containing vacuole (LCV) into the replicative vacuole (RV) recent studies propose at least two stages of development (Robinson & Roy 2006). The first stage is the recruitment and fusion of ER-derived early secretory vesicles with the nascent LCV. The second stage, which is dependent on the first stage, involves fusion with the ER to form the replication vacuole. After 4 h, the vacuolar membrane has thickened and is additionally decorated with ribosomes and even mitochondria. At this time the organism has begun to replicate and the organelle thus is termed the replication vacuole at this stage (Horwitz 1983). The rough ER does not seem to have any impact as a protein source for the bacteria. *L. micdadei* did not show rough ER association in any of the studied cell types as macrophages, epithelial cells and protozoa (Abu Kwaik 1998c, Abu Kwaik 2000).

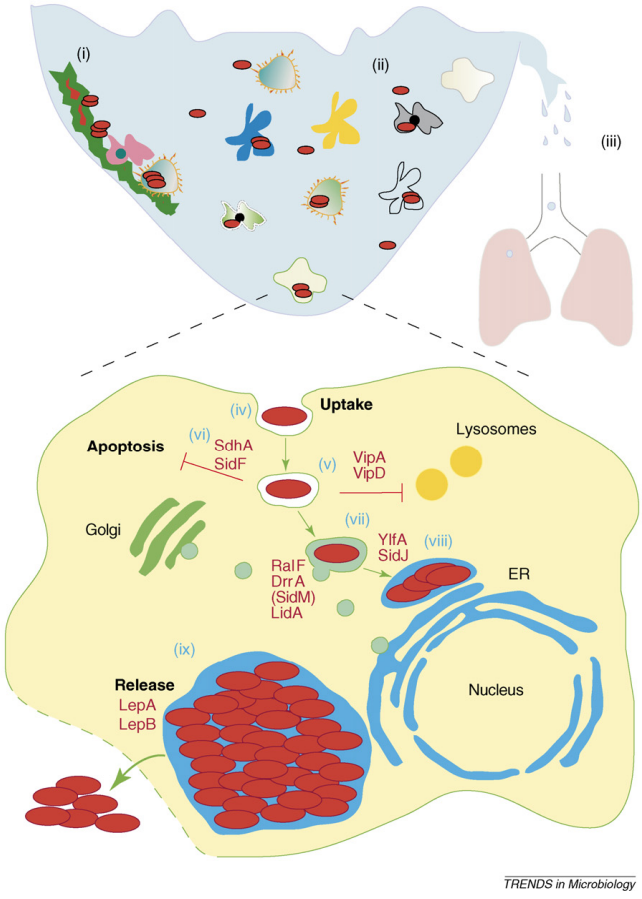

Fig. 1. 2. Life-cycle of *L. pneumophila*.
In the environment *L. pneumophila* (red ellipses) is found (i) in association with biofilm communities and (ii) in planktonic form. It may survive within the cell in a wide range of protozoan hosts (as indicated by the differently shaped cells within the blue area) and environmental conditions. (iii) Exposure of humans to aerosolised contaminated water can result in the infection of alveolar macrophages. (iv–ix) The intracellular-life cycle of *L. pneumophila* in eukaryotic host cells: (iv) In macrophages *L. pneumophila* can be internalised by a wortmannin insensitive pathway. (v) Once within host cells, the bacterium quickly evades delivery to lysosomes and (vi) can prevent host cell death in infected macrophages. (vii) The vacuole in which *L. pneumophila* resides undergoes remodelling by intercepting host vesicular traffic flowing from the ER to the Golgi-apparatus. (viii) The vacuole then undergoes fusion with vesicles coming from the ER and transforms into a specialised compartment that supports *L. pneumophila* replication. (ix) After several rounds of replication, the bacteria can leave the infected cell and go on to infect neighbouring cells. Dot/ Icm effectors involved in these processes are indicated in red (from Ninio & Roy 2007).

Several pathogens as for example *Agrobacterium*, *Bordetella*, *Helicobacter* and especially the intracellular pathogens *Coxiella*, *Brucella* and *Legionella* have evolved type IV secretion systems through which the bacterium is able to deliver modulating effectors into the host cell (Vogel *et al.* 1998, Segal & Shuman 1998b, Roy 1999, Vogel & Isberg 1999, Christie & Vogel 2000). Type IV secretion systems are membrane-associated transporter-complexes used to deliver substrate molecules to a wide range of target cells. One example would be horizontal DNA transfer to other bacteria and eukaryotic cells or DNA uptake from or even release into the extracellular milieu. Another function is toxin secretion and the injection of virulence factors into eukaryotic host target cells by several mammalian pathogens (Backert & Meyer 2006).

In the case of *L. pneumophila*, one type IV secretion system, the so-called Dot/ Icm (defective for organelle trafficking/ intracellular multiplication) (Marra *et al.* 1992, Berger & Isberg 1993), is essential for virulence and has thus been studied extensively. The Dot/ Icm system is necessary during phagocytosis to prevent phagosome–lysosome fusion and later on for establishment of the *L. pneumophila* replication vacuole (Matthews & Roy 2000, Brüggemann *et al.* 2006). Formation, but not the maintenance, of the LCV is dependent on the Dot/ Icm type IV secretory system (Coers *et al.* 1999). *DotA* deletion mutants show defects in intracellular replication and later host cell killing (Marra *et al.* 1992, Berger & Isberg 1993, Berger *et al*. 1994, Segal & Shuman 1998a) as well as less efficiency in uptake than wild type *L. pneumophila* (Hilbi & Segal 2001).

The gene-loci *dot* and *icm* are situated on two different 20 kb chromosomal regions and are assumedly transcribed in 9 operons (Segal *et al.* 1998, Vogel *et al.* 1998). It has been suggested that the products of the 24 *dot/ icm*-genes build a membrane associated complex leading to the insertion of a pore into the membrane of the eukaryotic host (Roy & Isberg 1997, Kirby *et al.* 1998, Kirby & Isberg 1998). Through this transportation-apparatus (transferosom) effector-molecules are secreted that modulate the endosomal pathway that otherwise would lead to phago-lysosomal fusion (Vogel *et al.* 1998, Roy 1999, Zuckman *et al*. 1999). Δ*dotA* mutants lead within a few minutes to accumulation of endosomal/ lysosomal markers e.g. GTP-binding protein Rab7 and LAMP-1 (lysosomal associated membrane protein 1) (Roy *et al.* 1997, 1998). The observation of the fast accumulation of these markers leads to the realization that the secreted effectors must be proteins and not DNA (Wiater *et al.* 1998). Bioinformatics and translocation assays have lead to the identification of more than 100 putative effectors, many showing multiple paralogous sites with 30-90 % amino acid identity. This accounts for the observation that single effector knock-out mutants usually do not produce a phenotype. This functional redundancy is of vital importance to the pathogen (Cazalet *et al.* 2004, Brüggemann *et al.* 2006). Most of these translocated proteins have not yet been characterised functionally, but recent investigations suggest their roles in modulating diverse host processes such as vesicle trafficking, autophagy, ubiquitination and apoptosis.
Several known effectors of the Dot/ Icm system that have been studied are found associated with the LCV after translocation. This has been demonstrated for LidA, SidC, SidM (DrrA) and SdhA (Conover *et al.* 2003, Luo & Isberg 2004, Bardill *et al.* 2005, Campodonico *et al.* 2005, Murata *et al.* 2006, Laguna *et al.* 2006, Ninio & Roy 2007).

LidA, one protein exhibiting properties expected for a translocated substrate of Dot/ Icm was shown to be important for maintenance of bacterial cell integrity: it associates with the phagosomal surface, promotes replication vacuole formation, and is important for intracellular growth (Conover *et al.* 2003). LidA is secreted early and stays expressed throughout the replication cycle. In most of the *L. pneumophila* strains, the loss of *lidA* does not cause as severe a defect in intracellular growth as exhibited by the *ΔdotA* mutant (Conover *et al.* 2003) indicating that the function of the protein may be partially redundant. When over expressed in mammalian cells or yeast, LidA interferes with the early secretory pathway, probably via a domain predicted to be rich in coiled-coil structure (Derré & Isberg 2005). Coiled-coil domains mediate protein-protein interactions either for protein multimerisation or for macromolecular recognition. Notably, all nine substrates (SidA-H, SdeC) of the type IV secretion system and LidA, LepA and LepB contain coiled-coil domains (Cazalet *et al.* 2004). LidA has been shown to preferentially bind to the GTP-bound form of Rab1 (Machner & Isberg 2006), but also binds to Rab6 and Rab8 (Machner & Isberg 2006). Although Δ*lidA* mutants still recruit Rab1 to the LCV, these mutants display a much more dramatic decrease in intracellular replication than did the Δ*sidM (drrA)* mutant (Machner & Isberg 2006). From this it was concluded that LidA targets multiple trafficking pathways, in opposition to SidM (DrrA), which targets only a single Rab1-dependent trafficking pathway.

SidC was shown to compete with another effector, SidM (DrrA), for anchoring to limiting amounts of Phosphatidyl-inositol (4) Phosphate (PI(4)P) on the vacuole (Brombacher *et al.* 2008). Phospho-inositides are phosphorylated by kinases at the positions 3, 4 or 5 of the D-*myo*-inositol ring and dephosphorylated by phosphatases, or hydrolysed by phospholipases to serve as second messengers or membrane anchors for effector proteins. PI(4)P is a component of vacuoles containing Dot/ Icm-proficient *L. pneumophila* (Hilbi 2006). By anchoring to the LCV via PI(4)P, SidC might directly engage host cell components to subvert vesicle trafficking or function as membrane anchor/adaptor proteins for other Dot/ Icm-secreted effector proteins.

In 2006 two groups independently identified the Dot/ Icm effector, SidM (DrrA), which localizes to the cytosolic face of the LCV and functions to recruit and regulate Rab1's activity at the LCV. In one study, SidM (substrate of Icm-Dot) was identified as a result of its ability to bind purified glutathione S-transferase–Rab1 (Machner & Isberg 2006). In a second study, DrrA (defect in Rab recruitment) was found in a mutant screen designed to identify *L. pneumophila* proteins that were necessary for the recruitment of Rab1 to the LCV (Murata *et al.* 2006). SidM as well as LidA is known to bind Rab1 but although the deletion of SidM (DrrA) prevents the recruitment of Rab1 to the LCV, no decrease in bacterial replication results from a loss of this protein (Machner & Isberg 2006, Murata *et al* 2006). Consistent with a collaborative relationship between the two proteins, immobilised SidM and LidA synergize to promote Rab1-dependent binding of early secretory vesicles (Machner & Isberg 2006).

Modulation of host signal transduction mechanisms

After learning about the above-mentioned effectors it has become an intriguing issue to see how exactly *L. pneumophila* subverts host functions to enter, survive, replicate and evade amoebae or alveolar macrophages. *L. pneumophila* has been shown to encode an abundance of eukaryotic-like proteins in its genome. Indeed, 30 genes encode proteins with high similarity to eukaryotic proteins,

and 32 genes encode proteins with eukaryotic domains that are implicated in protein-protein interactions (Cazalet *et al.* 2004). These proteins are characterised for example by ankyrin repeats, F-Boxes, U-Boxes or Sel-1 putatively enabling the pathogen to manipulate host cell functions as there are interaction with host cytoskeleton and manipulation of eukaryotic ubiquitination machinery (Brüggemann *et al.* 2006). Serine-threonine-kinases have also been found in the screen for eukaryotic like proteins. These will be referred to in chapter 1. 5.

Replicative phase

Following a lag-phase of 3-6 h *L. pneumophila* begins to replicate within the cell over a generation time of approximately 2 h. This long lag phase has been explained as being due to recruitment of host cell organelles or as a necessary adaptation period of *L. pneumophila* to the intracellular milieu (Shuman 1998, Abu Kwaik 1998a).

L. pneumophila gene expression varies depending on the intracellular environment. This led to assay the genes exclusively induced or repressed within the cell (Cirillo *et al.* 1994, Abu Kwaik & Pederson 1996, Susa *et al.* 1996, Abu Kwaik 1998d). By differential display PCR more than 50 genes could be identified. Among these MI-genes (macrophage induced) was the *eml* gene-locus (early stage macrophage induced locus) expressed during the first hours after infection of U937 cells. Upon deletion of *eml* intracellular replication was drastically reduced (Abu Kwaik & Pederson 1996). More examples for solely within the cell expressed genes are the 19 kDa GspA (global stress protein), a 20 kDa pyrophosphatase (*ppa*) and the gene locus *hel* showing homologies to cation-transporters (Abu Kwaik & Engleberg 1994, McClain *et al.* 1996, Abu Kwaik *et al.* 1997, 1998d).

Evasion from the host cell

Within their specialised replication vacuole, the bacteria replicate to high densities, resulting in host cell lysis after approximately 24 h and infection of neighbouring macrophages (Horwitz 1988, Shuman 1998). Lysis has been explained physically, by enhanced *L. pneumophila* motility („phenotypic switch“) (Rowbotham 1986, Byrne & Swanson 1998). Other methods of lysis as cytotoxic effects by accumulated metabolic products (for example NH_3 from the amino acid metabolism) or by the secretion of an up to now hypothetical cytotoxin or rather LPS (Rowbotham 1986, Shuman 1998) have been proposed. In primary mouse macrophages, the interaction with the lysosomal pathway is only delayed, as the replication vacuole eventually matures into an acidified vacuole in a process that resembles autophagy (Amer & Swanson 2005, Sturgill-Koszycki & Swanson 2000).

During intracellular growth *L. pneumophila* undergoes a „developmental switch“ characterised by a growth phase dependent phenotypic modulation (Vogel & Isberg 1999). At the passage into the post-exponential phase *L. pneumophila* grow salt-sensitive; develop osmolytic resistance, build the flagellum, become cytotoxic and infectious (Byrne & Swanson, 1998). Expression of this virulence-associated phenotype - a transgression from a replicative to a virulent form –is switched on by starvation signals mediated by accumulation of (guanosine 3´5´-bispyrophosphate) (Hammer & Swanson 1999). One factor known to activate such a switch is the alternative sigma-factor RpoS. A share of *rpoS* in this growth phase dependent stress resistance analogous to *E. coli* could not be

shown for *L. pneumophila*. Intracellular growth of such an *rpoS*-mutant in protozoa (*A. castellanii*) was not successful. Contrary to the protozoan host, the *rpoS*-deletion mutant was not impaired in its growth within macrophages (Hales & Shuman 1999a).

One interesting point is induced apoptosis of the host cell. It has been shown that *L. pneumophila* are able to induce apoptosis in macrophage-like cell lines HL60 and U937 as well as in peripheral blood monocytes and alveolar epithelial cells (Müller *et al.* 1996, Hägele *et al.* 1998, Abu Kwaik 1998b). Even living extracellular *L. pneumophila* induced apoptosis. On this basis, Gao and Abu Kwaik postulate that apoptotic effects within the host-cell are triggered by contact-mediated export of bacterial factors. These are expected to activate the signal-transduction-cascade - especially the activation of Caspase 3 –, which induces the programmed cell death of the host (Gao & Abu Kwaik 1999a, b, 2000). Cell death, however, is delayed by anti-apoptotic signalling of NF-κB as long as the bacteria replicate inside the cell. This mechanism again is dependent on a functioning Dot/ Icm secretion system, indicating the importance of its effectors on *L. pneumophila* survival (Banga *et al.* 2007). Only after completed replication does the cell undergo apoptosis.

1. 4 Cellular trafficking in *L. pneumophila* infection

Replication of pathogenic bacteria in eukaryotic hosts involves interactions between microbial virulence proteins and eukaryotic molecules at the surface of or inside host cells. The pathogens generally make use of existing cellular pathways that are designed for nutrient uptake, receptor down-regulation and signalling (Gruenberg *et al*. 2006). One such process is the eukaryotic secretory pathway, in which proteins and lipids are modified and transported from the endoplasmic reticulum (ER) through the Golgi apparatus network to the plasma membrane and to other cellular destinations. *Legionella*, *Brucella, Salmonella* and *Chlamydia* are known to engage steps of the pathway to establish intracellular replicative organelles (Fig. 1. 3).

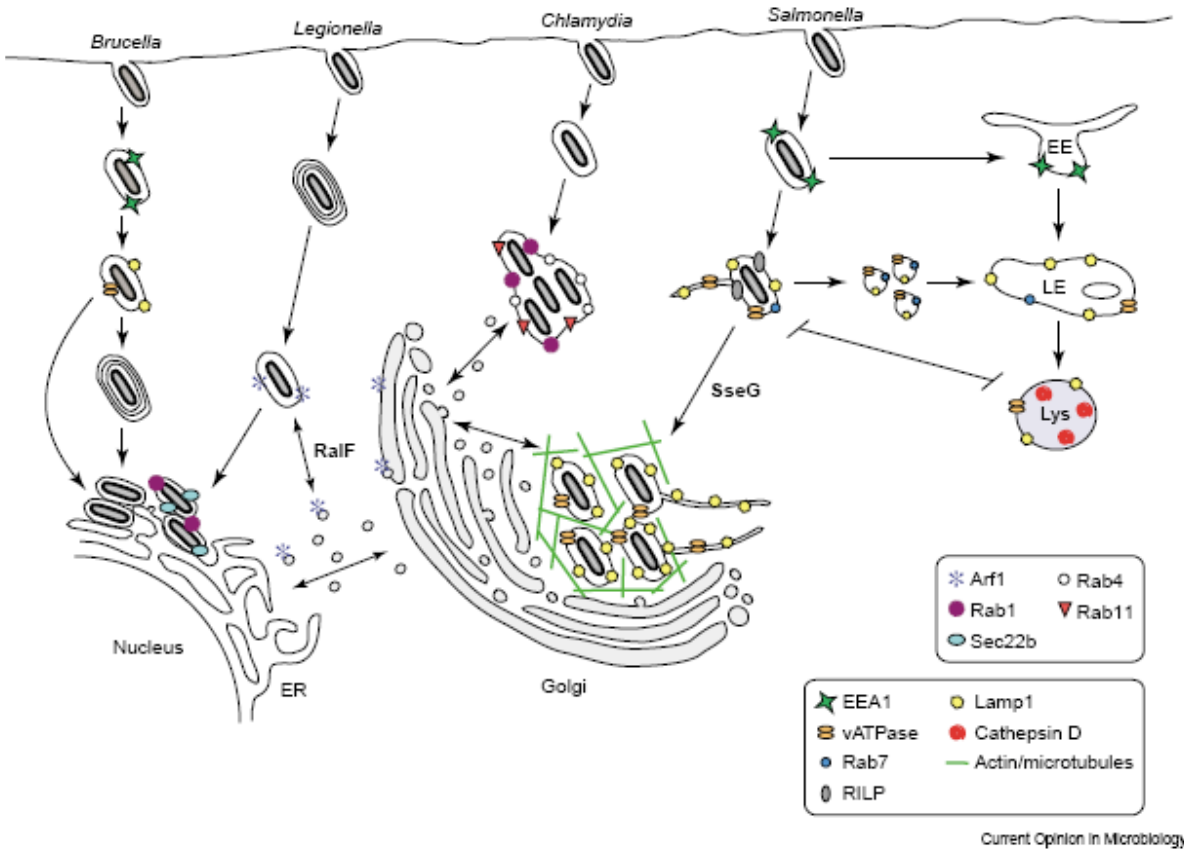

Fig. 1. 3. Intracellular bacterial pathogens interacting with the secretory pathway.
In macrophages, *Brucella* and *L. pneumophila* segregate from the endocytic pathway and replicate within a compartment characterised by the presence of endoplasmic reticulum (ER) markers. One report suggests that *L. pneumophila* can also transit through the autophagic pathway in macrophages (Swanson & Isberg 1995). The Dot/ Icm type IV secretion system effector RalF mediates recruitment of Arf1 onto *L. pneumophila*-containing vacuoles, which then accumulate Rab1 and Sec22b at ER exit sites. The VirB type IV secretion system mediates interactions between Brucella and the ER, but no effectors have yet been identified. *Chlamydia* does not interact with the endocytic pathway but instead localises in the vicinity of the Golgi apparatus, where it intercepts exocytic vesicles. By contrast, *Salmonella* undergoes selective interactions with the endocytic pathway in epithelial cells and macrophages (modified after Salcedo & Holden 2005).

Proteins in eukaryotic cells are translated by ribosomes. Some of these attach to the ER, where they translocate the growing amino acid chain into the lumen of the ER (Fig. 1. 4). Several proteins then are accumulated and sorted into vesicles that bud at ER exit sites and fuse to form the tubular vesicular ER–Golgi intermediate compartment (ERGIC). Clusters of the ERGIC then move along microtubules into the direction of the Golgi compartment where they fuse into the *cis*-Golgi network. The proteins then are passed on through the *cis*-, *medial*- and *trans*-cisternae of the Golgi apparatus stack. During their progress they can be glycosylated or undergo other post-translational modifications. From the *trans*-Golgi apparatus network (TGN) the vesicles are destined either to other cellular organelles or to the cell surface. Lipids as ceramides and cholesterol are also transported from the ER to the Golgi apparatus and later sorted in the TGN.

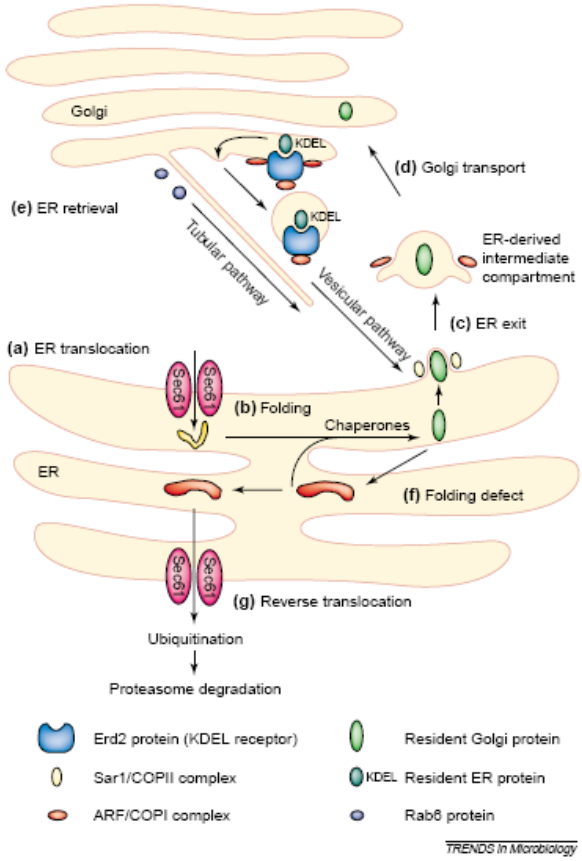

Fig. 1. 4. Protein import and export from the endoplasmic reticulum (ER).
(a) As they are translated, proteins with an amino-terminal signal sequence are translocated into the ER through a channel formed by the Sec61 complex. (b) Protein glycosylation occurs in the ER lumen and folding is facilitated by protein chaperones. (c) The Sar1/COPII system forms anterograde transport vesicles containing cargo proteins to be delivered to the Golgi apparatus. (d) ARF/COPI functions are required for transport of ER-derived vesicles to the Golgi apparatus. (e) Retrieval of proteins and membrane back to the ER is mediated by two complementary retrograde transport pathways. Rab6 controls formation of tubules that connect the Golgi apparatus with the ER. The Erd2 protein binds proteins with a carboxy-terminal KDEL motif and transports the cargo back to the ER in vesicles formed by the Arf1/ COPI system. (f) Defects in protein folding are detected in the ER and folding errors are either corrected by lumenal chaperones or proteins are targeted to the Sec61 complex for reverse translocation. (g) During reverse translocation, proteins are ubiquitinated, which targets them for proteasome-mediated degradation (modified after Roy 2002).

This transport from the ER to the Golgi apparatus is termed anterograde trafficking and is defined by the coat protein COPII. These coat proteins require the small GTPase Sar1 for vesicle formation. COPII and Sar1 together mediate the formation of the so-called “early secretory vesicles” at ER exit sites. There is growing evidence that COPII subunits contain multiple binding domains that recognise specific cargo, and mediate recruitment of different SNARE (see below) proteins (such as Sec22) onto ER-derived vesicles (Miller *et. al.* 2003, Mossessova *et al.* 2003). In the ERGIC the COPII coat is replaced by COPI. COPI coat proteins interact with the small GTPase Arf1 and its guanine nucleotide exchange factor GBF1 (Garcia-Mata *et al.* 2003). The vesicles then are transported to the *cis*-cisternae where they fuse with the Golgi apparatus.

The anterograde transport corresponds to the retrograde transport. This way of transport delivers misfolded proteins, ER chaperons and other proteins back to the ER. The transportation depends on COPI vesicles progressing on membrane tubules extending from the Golgi apparatus to the ER. Some of the above mentioned proteins contain a KDEL motif that enables the COPI-binding protein Erd2 (also known as the KDEL receptor) to retrieve them from the Golgi apparatus and deliver them to the ER (Aou *et al.* 1997). On the other hand, TGN-carriers sort proteins from the TGN to the plasma membrane or other cellular locations. These are clathrin-coated and un-coated vesicles and tubules (Gleeson *et al.* 2004). Some of the proteins transported to the Golgi apparatus undertake manifold functions in the Golgi apparatus network itself. They mediate tethering events and membrane fusion or provide structural support. Golgi apparatus structure, positioning and transport depends on a

complex cytoskeleton matrix composed of microtubules, actin, spectrin and intermediate filaments, along with molecular motors of the dynein, kinesin and myosin families (Allan *et al.* 2002).
Within this pathway the ER apparently provides a source of membranes during macrophage phagocytosis. Since ER proteins were found even on newly formed phagosomes containing latex beads, and on phagosomes inhabited by live pathogens such as *Leishmania* and *Salmonella*, it appears likely to consider the ER rather non-discriminating. The involvement of the ER in this process does not appear to depend on the nature of the particle being phagocytised (Gargnon *et al.* 2002). However a pathogen-specific engagement of the ER does occur after uptake of *L. pneumophila* and *Brucella* into host cells.

As mentioned above, *L. pneumophila* rapidly assembles an ER-like compartment shortly after entry into macrophages by hijacking vesicles from the early secretory pathway at ER exit sites (Tilney *et al.* 2001, Kagan & Roy 2002). This interaction is essential for intracellular bacterial replication (Kagan & Roy 2002) and has been shown to require the Dot/ Icm type IV secretion system (Roy *et al.* 1998). Recent studies have identified several Dot/ Icm translocated effector proteins, which have been referred to above (Nagai *et al.* 2002, Conover *et al.* 2003, Chen *et al.* 2004, Luo & Isberg 2004, Roy *et al.* 2006). It has been postulated that these proteins might be able to either mimic or disrupt the function of eukaryotic factors thus modifying the identities of their phagosomes in order to exploit host cell trafficking pathways and create an intracellular niche that favours bacterial replication.
Three translocated substrates of the *L. pneumophila* type IV secretion system interacting specifically with this early secretory pathway have been well characterised: the effectors RalF, SidM (DrrA) and LidA. One of the most extensively studied effectors, RalF, mediates recruitment of the small GTPase ADP-ribosylation factor 1 (Arf1), involved in retrograde vesicle transport from the Golgi-apparatus (GA) to the ER, to *L. pneumophila*-containing phagosomes (Nagai *et al.* 2002). RalF activates the GTPase by enabling exchange of GDP for GTP. This was verified by experiments using a dominant-negative Arf1 protein (in this course also inhibiting Golgi apparatus function) and by exposure of cells to Brefeldin A (Brefeldin A locks Arf1 in its inactive, GDP-bound state) which promptly inhibited bacterial replication. The *ΔralF*-mutants - no longer able to recruit Arf1 – still replicate in murine macrophages (Nagai *et al.* 2002).

Rab GTPases

Organelle identity is determined, in part, by the composition of active Rab GTPases on the membranes of each organelle. Proteins of the Rab GTPase family have regulatory roles in the formation, targeting and fusion of intracellular transport vesicles. Rab proteins belong to the Ras-superfamily of small guanine nucleotide binding proteins (GDPs) (Brumell & Scidmore 2008).
Rab proteins act as molecular switches, changing from their active GTP-bound state to the inactive GDP-bound state. Since the Rab proteins themselves do not have high intrinsic guanine nucleotide exchange or hydrolysis activities, other proteins work for Rab as guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) to see to their function. In their GDP-bound state, Rab´s are typically soluble and bound to a guanine nucleotide dissociation inhibitor (GDI). A GEF converts Rab to its GTP bound, active, conformation, allowing it to interact with its downstream

effectors (Fig. 1. 5). Through their effectors, Rab´s control many aspects of membrane traffic, including vesicle formation, vesicle motility along the actin/ microtubule cytoskeletons, vesicle tethering, transport, and fusion (Gurkan *et al.* 2005, Somsel Rodman & Wandinger-Ness 2000, Zerial & McBride 2001).

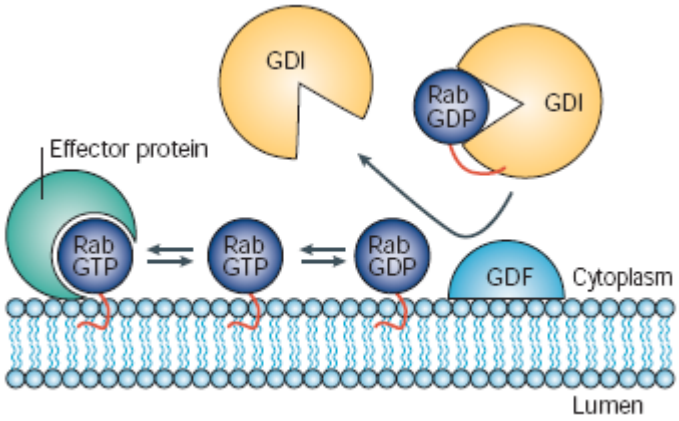

Fig. 1. 5. Rab GTPase switches.
Rab GTPases are present either on membranes or in the cytosol, where they are bound to their GDP-dissociation inhibitors (GDI). GDI only binds to GDP-bearing Rab proteins. Complexes of Rab proteins bound to their GDI contain all the information that is needed for accurate membrane delivery. The GDI displacement factors (GDFs) now recognize subsets of Rab proteins and catalyse their dissociation from the GDI at particular membrane surfaces. After membrane delivery, Rab proteins inter-convert between inactive, GDP- bound forms and active, GTP-bound forms. This interconversion is catalysed by guanine nucleotide-exchange factors (GEF not shown). In their now active, GTP-bound forms, Rab GTPases bind to their specific 'effector' proteins. Some Rab effectors stabilize the Rab protein in its GTP bound form (from Pfeffer & Aivazian 2004).

Pathogens are known to manipulate this pathway (Fig. 1. 6). One cellular protein hijacked by *L. pneumophila*-containing phagosomes is the small GTPase Rab1. Rab1A/ Rab1B and Rab2 are involved in decisive membrane docking in ER to Golgi apparatus vesicular trafficking (Nuoffer *et al.* 1994, Wilson 1994, Tisdale *et al.* 1992, Kagan *et al.* 2004) in combination with at least one SNARE complex (see below) composed of one v-SNARE (Sec22b) and three cognate t- SNAREs (Stx 5, Membrin, Bet1) (Hay *et al.* 1997, Xu *et al.* 2000). Rab1 promotes the fusion of ER-derived vesicles with pre-Golgi apparatus and Golgi compartments by recruiting effectors, such as p115 (Allan *et al.* 2000) and GM130 (Moyer *et al.* 2001) that are necessary for the tethering and fusion of vesicles and facilitates the pairing of v-SNAREs with cognate t-SNAREs.

The *L. pneumophila* effector SidM (DrrA) mimics a Rab1 guanine nucleotide-exchange factor (Machner & Isberg 2006, Murata *et al.* 2006) recruiting Rab1 to the *L. pneumophila*-containing vacuoles (LCV). LidA enhances this process. SidM can disrupt Rab1-mediated secretory transport to the Golgi apparatus causing its fragmentation, by competing with endogenous exchange factors to recruit and activate Rab1 on plasma membrane-derived organelles (Murata *et al.* 2006). The SidM-mediated Rab1 activation process has been divided into three stages: GDI (GTP-dissociation inhibitor) release from Rab1 accompanied by membrane insertion of SidM-bound Rab1; catalysis of nucleotide exchange in Rab1, and release of activated Rab1 by SidM to allow downstream ligand binding to GTP-Rab1 (Machner & Isberg 2007). So SidM is a protein that has both exchange- and dissociation-activity toward a Rab GTPase. This unique ability of SidM to link GDI displacement to Rab1 activation explains how the intravacuolar pathogen *L. pneumophila* can efficiently exploit host cell Rab1 even in the presence of GDI that naturally interferes with this process (Machner & Isberg 2007).

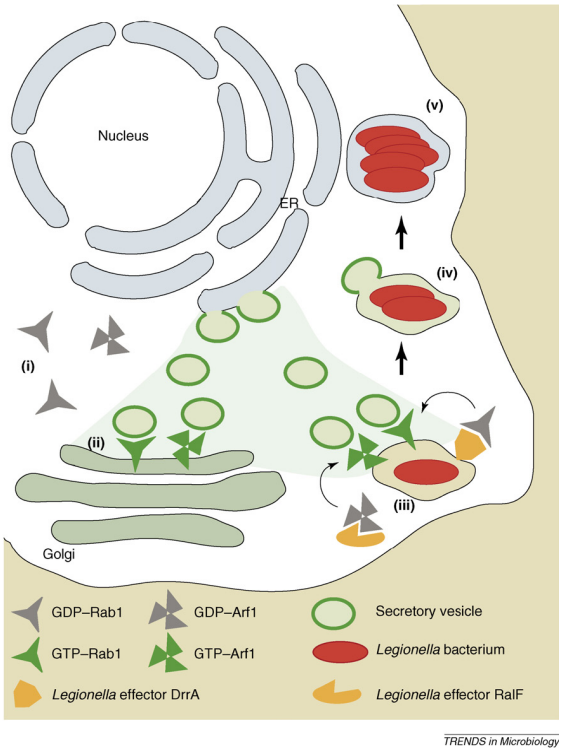

Fig. 1. 6. *L. pneumophila* employs translocated effectors to intercept host secretory vesicles.
The effectors RalF and SidM (DrrA) function as exchange factors for small GTPases Arf1 and Rab1, respectively. (i) Inactive GDP-bound Arf1 and Rab1 are found in the cytosol and can be activated by the host. (ii) In their activated form, they are associated with the *cis*-Golgi apparatus membrane, where they function in directing vesicular traffic originating in the ER towards the Golgi apparatus. (iii) *L. pneumophila* can mimic the *cis*-Golgi apparatus by activating host Arf1 and Rab1 on the bacterial vacuole. As a consequence, vesicles that are travelling from the ER towards the Golgi apparatus are effectively recruited to the *L. pneumophila* vacuole. (iv) This is followed by fusion of the vesicles with the vacuole. (v) The vacuole then undergoes fusion events with the ER and is further remodelled into an ER-like compartment that supports replication (from Ninio & Roy 2007).

In addition to Rab1, the sequential interactions of the two small GTPases, Sar1 and Arf1, which regulate the formation of COPII- and COPI-coated vesicles, respectively, are required for the production of early secretory vesicles (Aridor *et al.* 1995, Duden 2003, Scales *et al.* 1997). In *L. pneumophila* infection Arf1 as well as Rab1 recruitment is dependent on a functional Dot/ Icm secretion system. Rab1 acquisition was shown to be essential for intracellular replication. Upon activation GTP-Rab1 specifically interacts with downstream effectors such as the tethering protein p115 as well as other *cis*-Golgi apparatus proteins, thereby programming ER-derived vesicles for docking and fusion with the Golgi apparatus (Allan *et al.* 2000, Moyer *et al.* 2001, Weide *et al.* 2001, Diao *et al.* 2003). Succeeding Rab1 recruitment also the SNARE protein Sec22b accumulates on the *L. pneumophila*-containing phagosome by 1 h post infection as well as the resident ER marker calnexin by 4 h post infection as shown in immunofluorescence studies (Derré & Isberg 2004, Kagan & Roy 2002, Kagan *et al.* 2004). Sec22b is a known ER resident and might mediate further fusion events with ER-derived vesicles. The role of the Dot/ Icm effector proteins is essential in recruitment of components of host trafficking machinery onto the *L. pneumophila* vacuolar membrane.

SNARES

Synaptic vesicle´s membrane docking and fusion is mediated by SNAREs (soluble N-ethylmaleimide-sensitive factor attachment protein receptors) - located on the vesicle membrane (v-SNAREs) and on the target membrane (t-SNAREs). The SNARE hypothesis provides a mechanism for the specific docking and fusion of transport vesicles with their target membranes (Rothman & Warren 1994). SNAREs are small, abundant and mostly plasma membrane-bound proteins varying considerably in structure and size with a length of 100-300 amino acids. All share a segment in their cytosolic domain called a SNARE motif that consists of 60-70 amino acids, capable of reversible assembly into tight

bundles of four-helices, the so called *trans*-SNARE complexes. This homology domain is predicted to form a coiled-coil structure (Fig 1. 7B). The significance of this homology domain could be demonstrated by a partial suppression of the coiled-coil properties of the domain profile (Weimbs *et al.* 1997). The evolutionary conservation of the SNARE coiled-coil homology domain suggests that this domain has a similar function in different membrane fusion proteins. Coiled-coil domains are characterised by a heptad repeat pattern in which residues in the first and fourth position are hydrophobic, and residues in the fifth and seventh position are predominantly charged or polar. This pattern can be used by computational methods, to predict coiled-coil domains in amino acid sequences. In animals and yeast, the coiled-coil motif has been identified in a variety of proteins associated with the cytoskeleton, the Golgi apparatus, centromers, centrosomes, the nuclear matrix, and chromatin. These proteins play an important role in the association of functional proteins (e.g. signal transduction components) with the solid-state components of the cell.

Besides the functional classification into t- und v-SNAREs there also is a structural classification differentiating between Q- and R-SNARES (Fasshauer *et al.* 1998). Basic for this distinction is the homology of the SNARE region. R-SNARES contain arginine (R) central to the motif, in Q-SNARES this is a glutamine (Q).

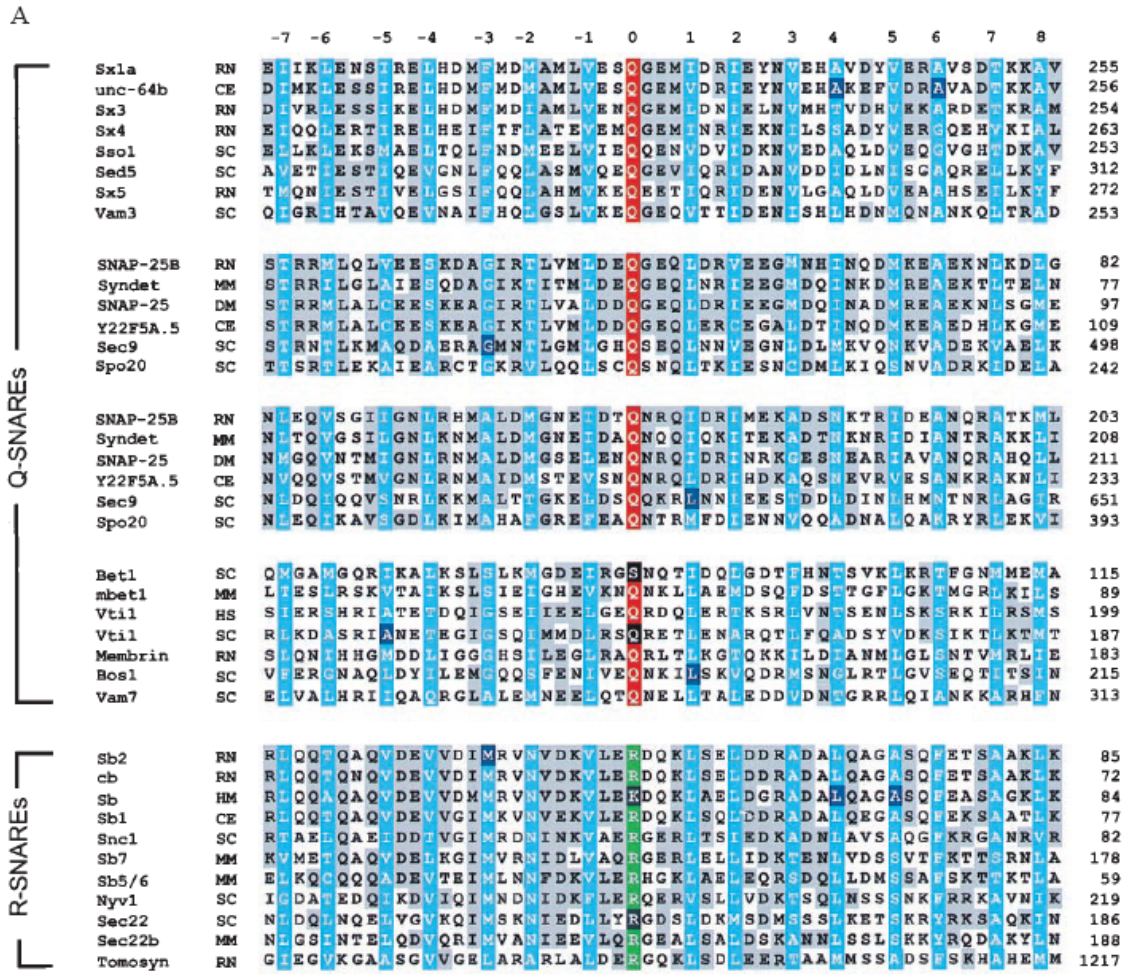

Fig. 1. 7. A Sequence alignment of the four-helix bundle region of a SNARE complex.
Blue depicts the four-helix bundle in the synaptic fusion complex. The conserved glutamine (Q) and arginine (R) residues forming the ionic 0 layer are indicated in red and green, respectively. This leads to the classification into Q- and R- SNARES.

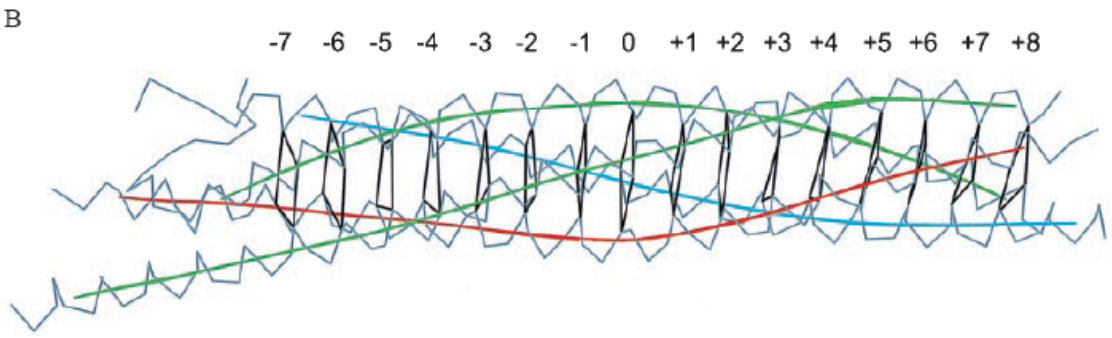

Fig. 1. 7. B Structure of the four-helix bundle region of a SNARE complex.
Gray indicates Ca traces; the helical axes are stained in blue, red, and green for synaptobrevin-II, syntaxin-1A, and SNAP-25b, respectively. The layers build virtual bonds between corresponding Ca positions - indicated by the black lines (modified from Fasshauer *et al.* 1998).

The assembled v-SNARE/ t-SNARE complex consists of a bundle of four helices, one of which is supplied by v-SNARE and the other three by t-SNARE (Rothman & Warren 1994, Sutton *et al.* 1998). Their interaction forms a core which is composed of a polar zero layer, a flanking leucine-zipper layer that acts as a water tight shield to isolate ionic interactions in the zero layer from the surrounding solvent (Fig 1. 7A). According to the "zipper" hypothesis, the complex assembly starts at the N-terminal parts of SNARE motifs and proceeds towards the c-termini that anchor interacting proteins in membranes assembling into stable membrane-bridging complexes. They gradually bring membranes in juxtaposition (Fasshauer 2002). Based on the stability of the resulting *cis*-SNARE complex, it has been postulated that energy released during the assembly process serves as a means for overcoming the repulsive forces between the membranes. This reaction delivers the energy needed to catalyse membrane fusion (Fasshauer 2003) (Fig. 1. 8).

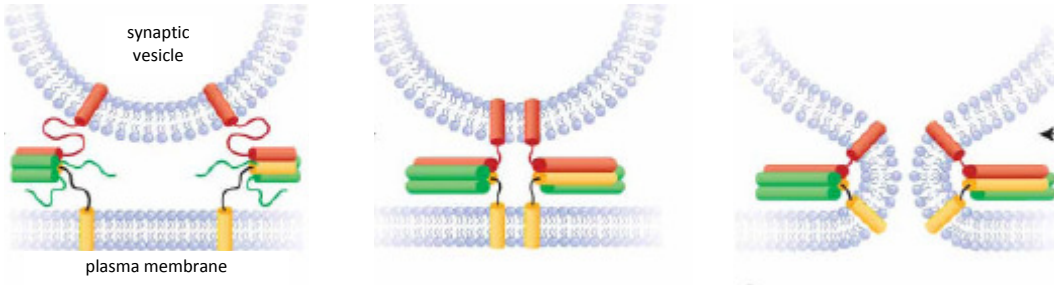

Fig. 1. 8. Schematic vesicle fusion via SNARE assembly.
Vesicle- and target-SNAREs are tethered together (left). They form a complex of α-helical bundles (centre) and induce membrane fusion as they fully assemble (right) (modified from Rizo & Rosenmund 2008).

There are several models that propose explanation for the subsequent step – the formation of stalk and fusion pore. The exact nature of these processes remains an open question. The activation energy of bilayer-bilayer fusion is very high (≈ 40 kBT). It was found that, in response to Ca^{2+} binding, synaptotagmin-1 could promote SNARE-mediated fusion by lowering this activation barrier while inducing high positive curvature in target membranes (Martens *et al*. 2007). Thus, synaptotagmin-1 triggers the fusion of docked vesicles by local Ca^{2+}-dependent buckling of the plasma membrane together with the zippering of SNAREs. To unlash the SNARE-helices for further reactions first the αSNAP (soluble NSF attachment protein) binds to the complex (Weimann *et al.* 1998). Secondly the

ATPase NSF (N-ethylmaleimide-sensitive factor) binds to αSNAP and releases the subunits under consumption of ATP (May *et al.* 2001).

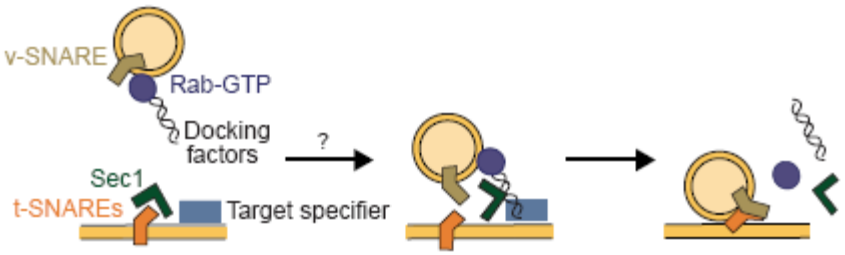

Fig. 1. 9. General logic of vesicular targeting and fusion. To target a vesicle derived from one type of organelle, such as the Golgi apparatus, to another, tethering and docking factors are recruited to nascent transport vesicles by an active (GTP-bound) Rab GTPase. GTP hydrolysis by Rab is involved in controlling the frequency of the docking process. On the target membrane, a target-specifying component recruits the vesicle by binding the vesicle-associated docking factor (left). Docking or other factors may then 'deprotect' or activate the t-SNARE (centre) to allow for t-SNARE–v-SNARE pairing (right) (from Pfeffer 1999).

One example for a tethering-fusion-interaction is the p115 tether-protein that conducts COPI vesicles to Golgi apparatus membranes (Fig. 1. 9). The acidic COOH-terminal domain of p115 links the golgins, Giantin on COPI vesicles, to GM130 on Golgi apparatus membranes. A SNARE motif-related domain within p115 stimulates the specific assembly of endogenous Golgi apparatus SNARE pins containing the t-SNARE, Syntaxin 5. p115 catalyzes the construction of a cognate GOS-28–syntaxin-5 (v-/ t-SNARE) complex by first linking the SNAREs (Shorter *et al.* 2002). This then promotes their direct interaction and subsequent fusion. These events have been shown in non-pathogenic context to be essential for example for NSF-catalysed reassembly of post mitotic Golgi apparatus vesicles and tubules into mature cisternae. The linking of golgins precedes SNARE pin assembly. Thus, p115 coordinates sequential tethering and docking of COPI vesicles by first using long tethers (golgins) and then short tethers (SNAREs).

Golgi apparatus

The Golgi apparatus is characterised by a series of flattened, cisternal membrane structures forming the heart of the secretory pathway. The Golgi apparatus stack is a polarised cellular compartment with a *cis*-face exchanging proteins and lipids with the endoplasmic reticulum (ER), and a *trans*-face communicating with the plasma membrane and compartments of the endocytic pathway. As secretory material passes through the Golgi apparatus in a *cis*- to *trans*- fashion, it becomes post-translational modified (for example glycosilated) in a sequential order before being sorted at the trans-Golgi-network (TGN) for delivery to its final destination within the cell. The organization of the Golgi apparatus is thought to depend on cytoplasmic structural proteins, including golgins, which form the Golgi-matrix (Shorter & Warren 2002). Golgins belong to a large family of proteins with coiled-coil domains that localize to the Golgi apparatus (Short et al. 2005).

Members of the golgin family of coiled-coil proteins have been implicated in the tethering of vesicles to Golgi apparatus membranes and cisternal membranes to each other. Many also bind to Rab GTPases. Golgin-84 for example is a membrane-anchored golgin that binds preferentially to the GTP form of the Rab1 GTPase (Diao *et al.* 2003, Satoh *et al.* 2003), as well as p115 has been reported to

bind to Rab1 GTP-dependent manner, suggesting that p115 is an effector protein of the Rab1 GTPase (Allan *et al.* 2000). Rab1B interacts with GM130 in a GTP-dependent manner (Weide *et al*. 2001). p115 also is known to directly interact with SNARE proteins that catalyze membrane fusion. Not only does p115 bind to certain early Golgi apparatus SNARE proteins but it was also showed to actually stimulate SNARE complex assembly, thereby linking initial membrane tethering to fusion (Shorter *et al.* 2002). The presence of Giantin in COPI vesicles and GM130 on Golgi apparatus membranes, and the ability of both proteins to bind p115, suggests a model in which COPI vesicles are linked to their target membranes by a Giantin–p115–GM130 ternary complex (Sonnichsen 2008).

These complex interactions are important during fragmentation of the Golgi apparatus during mitosis and apoptosis. During mitosis, fragmentation is triggered by the phosphorylation of several proteins, including golgin-84, GM130 and GRASP-65 as well as the inactivation of small GTPases. During apoptosis, fragmentation is induced by Caspase-dependent cleavage of golgins, such as Giantin, golgin-160 and p115 (Heuer *et al.* 2009).

Modification of the secretory pathway is an important aspect of the virulence of several bacterial pathogens. In future it might be possible to identify bacterial secreted proteins that enable intracellular bacteria such as *Legionella*, *Brucella* and *Chlamydia* to target the secretory pathway and establish their replication niche. This might become the basis for the development of novel ways to inhibit bacterial growth and for the identification of candidates for drug and vaccine design. The identification of the specific host cellular components targeted by these pathogens will additionally provide important insights into the molecular mechanisms mediating bacterial interference with the secretory pathway. In addition and not unimportantly this knowledge will increase our understanding of the normal functioning of the secretory pathway.

1. 5 Protein phosphorylation as a method of molecular control

The enormously complex interaction of the proteome within one cell demands highly specific regulatory mechanisms of activation and inactivation. The subsequent distinct interactions of the proteins given inside the cell thus create specific pathways. This regulation of protein activity is monitored by a great variety of ligands binding, including small organic molecules as there are lipids, carbohydrates, peptides, and proteins.

Simply adding a phosphate (PO_4^{3-}) molecule to a polar R group of an amino acid residue can turn a hydrophobic site of a protein into a polar and extremely hydrophilic portion of molecule. Thus a change in the structure of the protein via interaction with other hydrophobic and hydrophilic residues within the amino acid chain is effected. This is one way in which protein phosphorylation provides molecular control of complex physiological events within cells. Phosphorylation can also define protein fate by enabling interaction with specific "recognition domains", by definition of biological thermodynamics of energy-requiring reactions and by mediation of enzyme inhibition or even protein degradation. The idea that specific domains bind to distinct phosphorylated sequence motifs enlarges the possibilities of the cellular functions of proteins in breadth and diversity. In some proteins phosphorylation on tyrosine, serine or threonine residues creates binding sites for modular phospho-protein-binding domains, which connects these molecules with their upstream kinases and

downstream effectors to form multi-protein complexes that monitor their activity, binding partners or cellular localization. In addition, it provides a reversible way to regulate protein–protein interactions both spatially and temporally.

As mentioned above phosphorylation of proteins can modulate their activities by changing their 3D structure (Fig. 1.10). This has been demonstrated in several kinases as for example phospho-tyrosine kinases. These receptor tyrosine kinases (RTKs) catalyse transfer of the γ-phosphate of ATP to hydroxyl groups of tyrosines on target proteins (Hunter 1998). Phosphate is removed from phosphorylated tyrosines by enzymes called protein-tyrosine phosphatases. For example Src homology 2 (SH2) and phospho-tyrosine binding (PTB) domains specifically bind to phosphorylated tyrosine residues (Pawson & Scott 1997). Phosphorylation of Src by C-terminal-Src-kinase (Csk) induces a conformational change in the enzyme, resulting in a fold in the structure, which masks its kinase domain, and is thus “shut off".

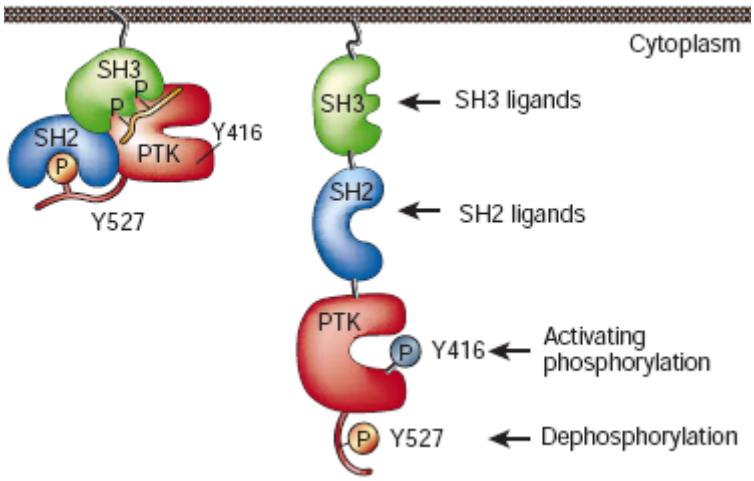

Fig. 1. 10. Molecular mechanism of c-Src activation.

Left: c-Src kinase activity is tightly repressed in the unstimulated state. The SH2 domain interacts with phospho-Tyr 527 in the C terminus and the SH3 domain with the polyproline type II helix in the linker region between the SH2 and kinase domain. This causes misalignment of residues that are critical for kinase activity. Right: binding of ligands to the SH2 or SH3 domain and/ or dephosphorylation of phospho-Tyr 527 by PTPs relieves the inhibitory constraints on the kinase, releasing the active kinase domain to allow substrate phosphorylation (from Blume-Jensen & Hunter 2001).

The MAPK-pathway is an example of a protein-protein interaction pathway in the eukaryotic cell induced by phosphorylation. The activated receptor tyrosine kinases recruit SH2- containing adaptor molecules such as Grb2. Grb2 then binds to receptor tyrosine kinases through its SH2 domain, but uses two SH3 domains to bind to the Ras guanine-nucleotide exchange factor son-of-sevenless (SOS), which facilitates the production of GTP-bound Ras and the subsequent activation of the mitogen-activated protein kinase (MAPK) pathway (Yaffe *et al.* 2002).

Infection of eukaryotic cells with bacteria alters host cell functions and protein phosphorylation might be a part of the associated molecular changes. Some pathogenic bacteria have evolved clever survival strategies for manipulating host cell signalling pathways to establish beneficial replicative niches within the host (protein phosphorylation being only one example of a broad range of tools by which effectors target key host proteins to remodel signalling events employed in a wide range of effects).

Many pathogens now hijack these protein interaction pathways by their own effectors mimicking cellular factors. One example is YpkA from *Yersinia* that targets the host cytoskeleton G protein signalling cascade. YpkA is involved in cytoskeletal rearrangements characterised by loss of actin stress fibres and consequent host cell rounding. It contains two major domains that function

synergistically: an amino-terminus resembling eukaryotic serine-threonine kinases of the PKA family and a carboxyl-terminus, containing a Rho-GTPase binding motif. Binding to actin at the carboxyl terminus activates the kinase of YpkA within the host cell. Once activated YpkA phosphorylates Gaq at Ser47, a novel phosphorylation site within its GTP binding fold, resulting in reduced affinity of Gaq for GTP, and thus disrupting the ability of Gaq to activate downstream signalling pathways (Hilbi *et al.* 2006, Mattoo *et al.* 2007). Another example of an effector targeting a completely different pathway would be *Shigella´s* OspF hijacking the MAPK pathway. OspF was shown to indirectly prevent the downstream-phosphorylation of histone H3 at the promoter for the pro-inflammatory cytokine IL-8. The lack of H3 phosphorylation causes it to mask NF-κB binding sites on the Il-8 promoter, thereby preventing transcription (Mattoo *et al.* 2007). Though most of the effectors mentioned above are targeting serine-threonine kinases either by mimicking or by direct targeting there is also IlnB, a *Listeria* internalin, presented on its surface and mediating up-take via the hepatocyte growth factor (HGF) receptor Met, a tyrosine kinase receptor. InlB acts as a ligand for this receptor and mimics the mode of action of the natural ligand, HGF, to cause phosphorylation of the Met receptor. This in turn activates PI(3)K and Rac-1, ultimately enabling bacterial internalization (Mattoo *et al.* 2007).

Although prokaryotes mainly exhibit phosphorylation of histidine, glutamic acid and aspartic acid residues, phosphorylation of proteins on serine, threonine and tyrosine residues is recognised as a key mode of signal transduction and amplification in eukaryotic cells (Hunter 1998). Phosphorylation on serine or threonine is much more common, than on tyrosine (1800: 200: 1). The rarity of tyrosine phospholylation makes it a higher gain in signalling because it is less abundant and more tightly regulated (Mann *et al.* 2002).

1. 5. 1 Intracellular communication via tyrosine phosphorylation upon *L. pneumophila* infection

The above-mentioned *L. pneumophila* virulence factors, especially the effectors translocated into the host cytosol have been shown to specifically interact with host proteins. Up until now we know of at least two specific ways by which *L. pneumophila* effectors interact with their host on basis of phosphorylation activities. First by manipulation of Rab-GTPase function (for example RalF exchanging Arf1´s Phosphate (Nagai *et al.* 2002)); second by modulation of phospho-inositide (for example SidC binding to PI(4)P on the vacuole (Luo & Isberg 2007)). By anchoring to the LCV via PI(4)P, SidC directly engages host cell components to subvert vesicle trafficking or function as membrane anchor/ adaptor-proteins for other Dot/ Icm-secreted effector proteins.

As one of the most abundant and important post – translational modifications in proteins, phosphorylation of specific protein sites leads to activation or blocking of the respective protein. This is a very viable and frequently used method of pathogens to disrupt cellular functioning. The knowledge that the cellular GDP Rab1 is an important factor in *L. pneumophila´s* interception of host trafficking led to search for an especially abundant phosphorylated protein.

It is known that *L. pneumophila* infection (at its entry) results in rearrangement of the cytoskeleton of its protozoan host (Venkataraman *et al.* 2000). In 1998 Coxon *et al.* found in MRC-5 cells that this

effect is specifically due to phosphorylation of proteins at the tyrosine residue especially of the tyrosine kinase (PK) and the protein kinase C (PKC). During late infection proteins phosphorylated on tyrosine residues accumulate progressively around or in phagosomes filled with bacteria. This specific association of tyrosine-phosphorylated proteins with the LCV was shown by Susa *et al.* 1999. They conclude a dysregulation of the cellular phosphorylation and dephosphorylation cascade supporting intracellular survival and replication of the pathogen. Yamamoto *et al.* 1992 examined protein phosphorylation in peritoneal macrophages from susceptible A/ J mice infected with *L. pneumophila* and other bacteria but only found induction of phosphorylation of a 76- kDa protein only in the *L. pneumophila* infected mice.

1. 6 Anti-apoptotic NF-κB-signalling in *L. pneumophila* infection

Many pathogens manipulate host signalling to promote their own survival. *L. pneumophila´s* ability to replicate within the cell depends on its survival inside the cell in addition to the cells survival until bacterial replication is completed. Here again the type IV secretion system translocating bacterial proteins into the host cell, is crucial. There are not many factors characterised yet, but two distinct effectors are known to particularly be involved in inhibiting macrophage cell death: SdhA and SidF (Laguna *et al.* 2006, Banga *et al.* 2007). Up to now *L. pneumophila´s* specific interaction with the anti-apoptotic pathway controlled by the mammalian nuclear transcription factor-κB (NF-κB) is well known. But how exactly the pathogen manipulates this way of action remains elusive. NF-κB stands for a family of transcription factors that link extracellular stimuli to cellular responses. These control inflammation, innate immunity, cell division and survival (Karin & Greten 2005, Hoffmann & Baltimore 2006, Hayden & Ghosh 2008). In *L. pneumophila* infection specific activation of NF-κB is dependent on the functional Dot/ Icm secretion system. This leads to the hypothesis that one or more of the secreted effectors may be involved in activation of the NF-κB regulated pathway.

1. 6. 1 NF-κB

NF-κB was originally detected as a regulator of expression of the κB light chain in B cells (so called nuclear factor "κ-light-chain-enhancer"). It is an important inducible transcription factor, present in almost all cells and tissues. By binding of the κB motive to ~10 bp enhancer regions on the nuclear DNA it positively or negatively influences gene transcription. NF-κB targets about 200 different genes and modulates varied effects. This pleiotropic signaling system includes signalling pathways emanating from many receptors, as for example the inflammatory tumor necrosis factor and Toll-like receptor super families. Two different pathways have been suggested: the non-canonical pathway is activated by a subset of TNF super family members, while the canonical pathway is activated by a broader and overlapping array of receptors. Current knowledge of the crucial part of the NF-κB-signalling system describes about a dozen different dimers comprising five homologous proteins: p65/ RelA, p50, p52, RelB, and cRel. These subunits form homo- and heterodimers. The most frequent combination of the canonical- or "classical-pathway" is p50-p65/ RelA (Fig 1. 11).

A wide range of different signals activates these receptors, as for example: pathogen –derived substances, inflammatory inter-cellular signals, developmental intracellular signals, environmental stress or metabolic stress. This manifold activation again induces different reactions triggered by the NF-κB system, there being: inflammation, survival, proliferation differentiation, apoptosis and cell cycle arrest.

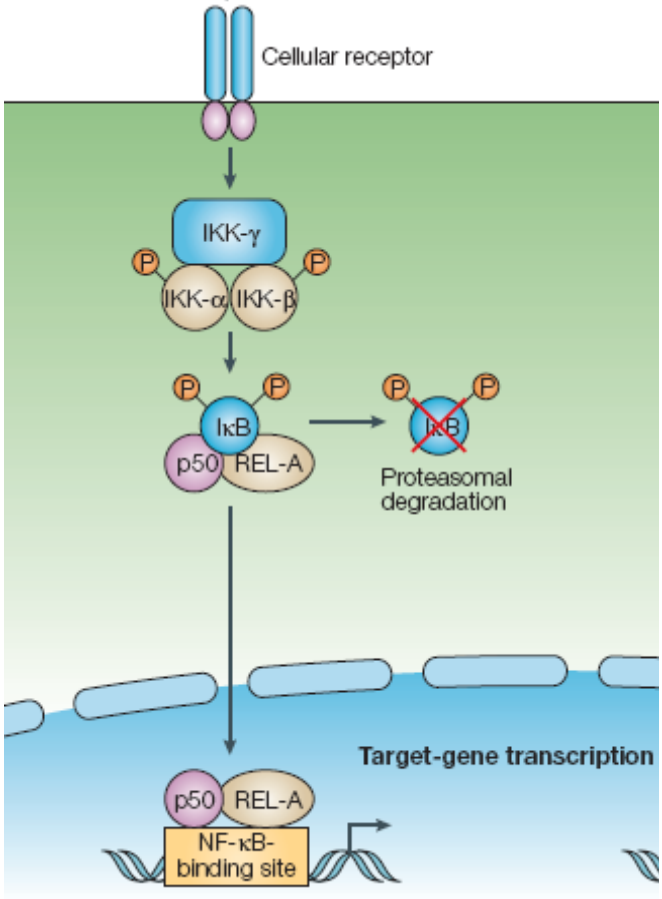

Fig. 1. 11. Signalling pathway after NF-κB activation.
NF-κB pathway represented here by the so-called IKK-IκB-NF-κB-signalling module mediates a wide variety of extracellular and intracellular signals to control a diverse set of cellular responses. Negative feedback control mediated by IκBα and IκBε allows for dynamic regulation of NF-κB. The classical pathway is triggered by pro-inflammatory stimuli and genotoxic stress including cytokines as Tumor Necrosis Factor (TNF) or Interleukin (Il-1); bacterial cell-wall components such as lipopolysaccharides, viruses and DNA-damaging agents. This leads to the IKKβ- and IKKγ-dependent phosphorylation of IκB´s resulting in their ubiquitination and proteasomal degradation, and to the subsequent liberation of the NF-κB dimer (Karin & Greten 2005).

The canonical pathway is described as follows. The NF-κB dimer is primarily bound to its inhibitor IκB. In the "classical pathway" the IκBα protein (specific inhibitor in case of p65/ RelA-p50) masks the nuclear localization sequence (NLS) of p65/ RelA, whereas the NLS of p50 remains exposed. The exposed NLS of p50 coupled with nuclear export sequences (NES) in IκBα and p65/ RelA leads to constant shuttling of IκBα-NF-κB-complexes between the nucleus and the cytoplasm (Ghosh & Karin 2002). Degradation of IκBα then drastically alters the dynamic balance between cytosolic and nuclear localization signals to favor nuclear localization of NF-κB. Degradation of IκB is a rapidly induced signaling event that is initiated upon specific phosphorylation of these molecules by activated IKK. An active phorbol ester activated by a protein kinase C causes ubiquitination of IκBα and subsequent dissociation of the cytoplasmic NF-κB-IκB-complex. The so marked IκB is degraded via the proteasome and thereby liberates the NF-κB dimer. The released phosphorylated active NF-κB dimer then translocates into the nucleus where p65/ RelA binds to the κB sites within the promoters/ enhancers of target genes and regulates transcription through the recruitment of co-activators and co-repressors (Baeuerle & Baltimore 1988). Activation of the classical pathway leads to increased transcription of genes that can be divided into three functional classes: inflammation and differentiation (COX2, TNF, IL-6), innate

immunity and proliferation (cytokines, chemokines, proteases and adhesion molecules) and survival (BCL-XL).

NF-κB has a central role in inflammation and innate immunity by regulating the expression of the molecules that enable cells to communicate with each other (cytokines and chemokines, cell surface receptors and adhesion molecules). This allows the coordination of the multipronged pathogen-specific human immune response that culminates in development of adaptive immunity. Therefore NF-κB signaling needs to be tightly controlled. This is administered by several negative feedback systems. One mechanism is the inhibitor IκBα itself being rapidly synthesised after NF-κB targeted its sequence. Although normally found in the cytoplasm, IκBα, newly synthesised in response, is transported to the nucleus. Here again IκBα associates with the p50 and p65/ RelA subunits of NF-κB, inhibiting DNA binding of the transcription factor (Arenzana-Seisdedos *et al*. 1995). NF-κB regulation of IκBα transcription represents a delayed negative feedback loop that drives oscillations in NF-κB translocation. Nelson *et al.* 2005 even went as far as to state that the functional consequences of NF-κB signaling might thus depend on number, period, and amplitude of oscillations. Although tumor necrosis factor–α (TNFα)– induced IKK activity was rapidly attenuated by negative feedback, lipopolysaccharide (LPS) signalling and LPS-specific gene expression programs were dependent on a cytokine-mediated positive feedback mechanism for stimulus specific gene expression (Werner *et al.* 2005). The IKK complex contains two highly homologous kinase subunits, IKKα/IKK1 (CHUK) and IKKβ/IKK2 (IKBKB), and a regulatory subunit NEMO (NF-κB essential modulator)/IKKγ (IKBKG) (Häcker & Karin 2006). So, another mechanism including the three isoforms of the inhibitors IκBα, IκBβ and IκBε leads to dampening of the oscillations by delayed transcription of IκBε, relative to that of IκBα, rendering the two negative feedback loops to be in antiphase (Hoffmann *et al.* 2002, Kearns *et al.* 2006). Another important node of the NF-κB pathway is its control by the ubiquitin (Ub) system at many levels. Ub-conjugation targets upstream signalling mediators as well as nuclear NF-κB to post-inductive degradation to limit the duration of signalling (Krappmann *et al.* 2005). Additionally after activation, p65/ RelA is degraded by the proteasome in the nucleus and in a DNA binding–dependent manner (Saccani *et al*. 2004).

One consequence of so many pathways converging on one transcription factor, however, is that dysregulation of its function can have a broad deleterious impact. As an example continuous nuclear localization of NF-κB subunits is found in many cancers (reviewed in Gilmore 2006). This is easily explained by the fact that permanent nuclear localisation of NF-κB results in continuous activation of anti-apoptotic genes. This then leads to the un-controlled cell growth, cancer is characterized of.

1. 6. 2 Biphasic NF-κB activation upon *L. pneumophila* challenge

L. pneumophila now is known to induce nuclear translocation of NF-κB in infected macrophages. First *L. pneumophila* were detected to activate caspase-3 one of the tree effector caspases of apoptosis in macrophages after infection (this not holding true in protozoan hosts). But surprisingly the cells were still not prone to apoptosis until the bacteria had sufficiently replicated (> 20 bacteria in macrophages). Abu-Zant *et al.* 2007 then linked activation of NF-κB to this anti-apoptotic behaviour.

In micro-array analyses they found a distinct set of genes up regulated upon infection, all controlled by NF-κB. Subsequent experiments showed infection dependent translocation of active p65/ RelA to the nucleus. This host cell reaction is again triggered in a Dot/ Icm dependent manner (Laguna *et al.* 2006, Brüggemann *et al.* 2006, Ninio & Roy 2007, Kubori *et al.* 2008). NF-κB is activated permanently so that the natural oscillations in and out of the nucleus can no longer be observed. Losick and Isberg 2006 had shown a dose dependent differing activation of NF-κB by *L. pneumophila*. Low dose infections lead to Dot/ Icm-dependent and MyD88- independent NF-κB activation, whereas at highter multiplicitys of infection (10-fold), signaling occurred via MyD88 (TLR-5 dependent activation) and in a Dot/Icm independant manner. The activation of MyD88 has been found to be important for secretion of early inflammatory cytokines and host protection in response to *L. pneumophila* infection, subsequently leading to pulmonary clearance of the pathogen (Archer *et al.* 2008). *L. pneumophila* infection of human lung epithelial cells (Cianciotto *et al.* 1995, Gao *et al.* 1998) leads to secretion of cytokines such as interleukin (IL) 8 in a NF-κB dependent manner thereby promoting the disease (Chang *et al.* 2004, Schmeck *et al.* 2007). The low dose NF-κB activation could be further characterised by Il-8 production. This again was linked to the pathogen´s flagellin, as flagellin deficient mutants (*ΔflaA*) were no longer able to induce Il-8 production, though they were still able to activate NF-κB (Bartfeld *et al.* 2009).
Mutants lacking the Dot/ Icm secretion system were not able to permanently locate p65 to the nucleus. A short activation of the NF-κB pathway could be observed with these mutants during the first 90 min, due to the flagella mediated TLR5 – MyD88 pathway. From this, Bartfeld *et al.* 2009 propose a dependence of the permanent NF-κB activation upon *L. pneumophila* infection on a functional Dot/ Icm system and its effectors. This made it an interesting task to investigate effectors on this specific trait.

1. 6. 3 *L. pneumophila* virulence traits in NF-κB activation

Two different virulence traits have been identified to be involved in NF-κB activation in *L. pneumophila* infection. One is the bacterial flagellum; the other factor is connected with the Dot/ Icm secretion system. Schmeck *et al.* showed in 2006 that flagellin of the pathogens flagellum is able to induce interleukin-8 (Il-8) secretion. Il-8 is a chemokine produced by epithelial cells. It activates neutrophils, basophils and T cells inducing chemotaxis and granula expression and is involved in the early host response to pathogens (Eckmann *et al.* 1993). Flagellin is recognised by TLR5 (Hawn *et al.* 2003), and NF-κB signaling depends on the TLR adaptor protein MyD88 (Losick *et al.* 2006, Teruya *et al.* 2007).
The other feature that enables *L. pneumophila* to activate the NF-κB pathway is its Dot/ Icm secretion system. This is essentially needed for up regulation of anti-apoptotic genes in infections of human macrophages (Abu-Zant *et al.* 2006, Losick *et al.* 2006).

1. 6. 4 Specific anti-apoptotic effectors of *L. pneumophila*: SidF, SdhA and SdbA

For establishment of the *L. pneumophila*-containing vacuole (LCV) the pathogen needs to translocate its effector molecules via the Dot/ Icm type IV secretion system into the host cells cytosol. As mentioned above most of these effectors could not yet be sufficiently characterised still leaving to suggestion their function in modulation of cellular processes in the hosts cytoplasm raging from vesicular trafficking over autophagy, phosphorylation and ubiquitination to apoptosis or anti-apoptosis which they both seem to interfere with (Banga *et al.* 2006, Laguna *et al.* 2006, Abu-Zant *et al.* 2007). On the one hand side it is known that *L. pneumophila* activate caspase-3, one of the effector caspases in apoptosis but on the other hand these cells still show anti-apoptotic markers (Gao & Abu Kwaik 1999, Molmeret *et al*. 2004b, Abu-Zant *et al*. 2005). Banga *et al.* 2006 succeeded in linking the Dot/ Icm substrate SidF to the inhibition of infected cells from undergoing apoptosis, allowing maximal bacterial multiplication. They found that cells expressing SidF were resistant to apoptotic stimuli. SidF contributes to apoptosis resistance in *L. pneumophila*-infected cells by specifically interacting with and neutralizing the effects of BNIP3 and Bcl-rambo, two pro-apoptotic members of Bcl2 protein family (Banga *et al.* 2006). This is the first account of a *L. pneumophila* effector that actively neutralizes the effect of the pro-apoptotic proteins.

Another effector protein surfaced when its deletion mutant showed impaired replication on macrophages. It is not usually perceived of *L. pneumophila* effectors that only one protein has such a great impact for most of these seem be redundant in function. The more surprising it was, to find that *L. pneumophila* mutants lacking *sdhA* to be severely reduced in replication and a triple mutant of all *sdhA* homologues could not replicate at all (Laguna *et al.* 2006). Additionally the host cell harbouring an *sdh*A mutant showed severe marks of apoptosis as for example increased nuclear degradation, mitochondrial disruption, membrane permeability, and caspase activation. This was taken to indicate a role for SdhA in preventing host cell death. In our set up we compared *L. pneumophila* replication of mutants lacking SdhA to ones lacking the Dot/ Icm in A549 cells. Here too the effector lacking *L. pneumophila* showed impaired replication whereas Dot/ Icm deficient *L. pneumophila* did not replicate at all. Even in the p65/ RelA translocation assay by Bartfeld *et al.* 2009 the mutant did not activate the system. Luo and Isberg shortly mention in 2007 that the *sdbA* mutant seemed to be deficient in growth in macrophages but did not further characterize this discovery.

Since all IκB proteins are characterised by the presence of multiple ankyrin repeat domains it might also be suggested that some of the ankyrin-repeat (Ank)- effector proteins mentioned above (Pan *et al.* 2008, Al-Khodor *et al.* 2008 and Habyarimana *et al.* 2008) might also be involved in the anti-apoptotic NF-κB signaling.

2 Material and Methods

Material

2. 1 Equipment

Shaking incubator G25	New Brunswick Scientific
Luminescent image analyzer LAS-3000	Fujifilm Life Science
Centrifuge 5417C	Eppendorf
Centrifuge 5417R	Eppendorf
Sorvall® RC-5B	Kendro Laboratory products
Confocal laser scanning microscope TCS SP	Leica
Stereo microscope SZ-60	Olympus, Hamburg
DIC light microscope IX-50	Olympus, Hamburg
Mini-PROTEAN® III Electrophoresis Cell	BioRad
Western Blot device Fastblot B33	Biometra
HERA cell 150 incubator	Heraeus
Photometer DR/200	Hach
Spectral photometer UltraSpec 3000	Amersham Biosciences
Nano drop Spectrophotometer ND-1000	PeqLab Biotechnologie
DUOMAX 1030 shaker	Heidolph
Abi Prism 7900HT	AME Bioscience
OPTIMAX 2010 X-Ray Film Processor	Protec Medizintechnik

2. 2 Material

Amersham Hyperfilm™	GE Healthcare
PVDF Transfer Membrane	PerkinElmer
Whatman chromatography paper	Schleicher and Schüll
Reaction tubes	Sarstedt
Falcon tubes	Sarstedt
Glass vessels	Schott
Microscope slides	Marienfeld
12 mm cover slips	Roth
Cell culture dishes	TPP and Corning Life Sciences
Sterile pipettes	Corning Life Sciences

2. 3 Software

Adobe Acrobat 7.0	Adobe
Image Reader LAS-3000	Fuji Film Science
Mozilla Firefox	Microsoft
Microsoft Office Word	Microsoft
Microsoft Office Exel	Microsoft
NCBI BLAST	NCBI
Photoshop 7.0	Adobe
Reference Manager 11	Thompson ISI research soft
Scan^{R}-Programm	Olympus
TCS	Leica

2. 4 Organisms

2. 4. 1 Cell culture

Cell line	Specification	Additional properties	ATCC
A549	Human lung-epithelial cells		CCL-185
A549SIB01	Human lung-epithelial cells	p65-eGFP-vector	
AGS	Human gastric adeno-carcinoma		CRL-1739
AGS- pDsred2-ER	Human gastric adeno-carcinoma	Stably transfected with vector pDsred2-eER (# 632409 Clontech Laboratories, Inc)	
HeLa	Human cervix adeno-carcinoma		CCL-2

ATCC: American Tissue Culture Collection (www.atcc.org)

2. 4. 1. 1 Media for cell culture

Medium	Supplements	Cell line	Supplier
DMEM	+ 10 % FCS + 1 mM Na-Pyruvate +1 mM Glutamine	A549, AGS	Gibco
RPMI 1640 (+ HEPES/ + Glutamin)	+ 10 % FCS	HeLa	Gibco
	+ 10 % FCS + 250 µg/ ml G418	AGS pDsred2-er	Gibco Invitrogen

2. 4. 1. 2 Supplements for cell culture

Solution	Supplier
D-PBS	Gibco
FCS (heat inactivated)	Biochrom AG
Genaticin (G418)	Invitrogen
Trypsin-EDTA solution	Invitrogen

2. 4. 2 Bacteria

***L. pneumophila* strain**	**Mutation in gene (wild type = wt)**	**Resistance**	**Reference**
Philadelphia-1	wt		Brenner *et al.* (1979)
Philadelphia-1 Δ*sdbA*	Lpg0275	Km	E. Siegbrecht, A. Flieger (unpublished)
Philadelphia-1 Δ*sdhA*	Lpg0376	Km	E. Siegbrecht, A. Flieger (unpublished)
Corby	wt		Jepras *et al.* (1985)
Corby Δ*flaA*		Km	Dietrich *et al.* (2001)
JR32	wt		Sadosky *et al.* (1993)
JR32 Δ*dotA*	Lpg2686 (Lela3118)	Km	Sadosky *et al.* (1993)
Paris	wt		Lawrence *et al.* (1999)

2. 4. 2. 1 Antibiotics

Antibiotic	Concentration
Kanamycin	10 µg/ ml
Gentamicin	5-10 µg/ ml

2. 5 Chemical reagents

(Chemicals not listed below were purchased from Roth, Merck, Serva and Sigma-Aldrich)

Chemical	Supplier
Complete	Roche
ECL substrate solution	PerkinElmer
FCS	Biochrom
Glycine	Biomol
LB Agar base	Invitrogen
Proteose Peptone	Becton Dickinson
Sucofin low-fat milk powder	TSI
Tris (hydroxymethyl)-aminomethan	AppliChem
Prestained Marker	NEB

2. 6 Antibodies

Primary antibodies

Antibody	Origin	Dilution	Source	Order No.
β-actin	Mouse	1:5000 (IB)	Sigma-Aldrich	A5441
Arf1	Mouse	1:1000 (IB)	ALEXIS	M275
ERGIC-53	Mouse	1:100 (IF)	Hauri (Basel)	
Giantin	Rabbit	1:5000 (IB) 1:500 (IF)	ALEXIS	G1/ 133
GM130	Mouse	1:5000 (IB) 1:500 (IF)	BD Bioscience	610823
Golgin-84	Mouse	1:1000 (IB)	BD Bioscience	
L. pneumophila	Rabbit	1:1000 (IB) 1:100 (IF)	Abcam	168437
L. pneumophila	Mouse	1:1000 (IB) 1:100 (IF)	Santa Cruz	L1506
NSF	Mouse	1:2000 (IB)	Oncogene	T021
Phospho-Tyrosine	Mouse	1:1000 (IB)	Cell Signaling	9416
Rab1A	Rabbit	1:1000 (IB)	Santa Cruz	FL205
Syntaxin5	Rabbit	1:1000 (IB) 1:100 (IF)	Santa Cruz	K0105
VAMP4	Rabbit	1:1000 (IB)	Sigma-Aldrich	T023

IB (Immunoblot); IF (Immunofluorescence)

Secondary antibodies

Antibodies Immunoblot (IB)	Dilution	Supplier
ECL™ donkey α-rabbit IgG, HRP-linked	1:3000	GE Healthcare
ECL™ sheep α-mouse IgG, HRP-linked	1:3000	GE Healthcare

Antibodies Immunofluorescence (IF)	Dilution	Supplier
Cy™2-conjugated goat α-rabbit IgG	1:150	Jackson ImmunoResearch
Cy™2-conjugated donkey α-mouse	1:150	Jackson ImmunoResearch
Cy™3-conjugated goat α-rabbit IgG	1:150	Jackson ImmunoResearch
Cy™3-conjugated donkey α-mouse	1:150	Jackson ImmunoResearch
Cy™5-conjugated goat α-rabbit IgG	1:150	Jackson ImmunoResearch
Cy™5-conjugated donkey α-mouse	1:150	Jackson ImmunoResearch

2. 7 siRNA

Protein	DNA - target sequence (xyN19)	Knock-down %	Source
Arf1[-2]	CACCATAGGCTTCAACGTGGA	95,6 %	Quiagen
Arf1[-r]	ACGTGGAAACCGTGGAGTACA	96,9 %	Quiagen
Bet1[-1]	CACTGATGTCGTGGCGCTTTA	77,0 %	Quiagen
Calreticulin	CAGTATCTATGCCTATGATAA	98,1 %	Quiagen
CASP[-1]	CAGCGCCTGCACGATATTGAA	87,4 %	Quiagen
CASP[-1r2]	CAGCGCCTGCACGATATTGAA	88,1 %	Quiagen
CASP[-2]	AAGGAATTTGCTGAAGTGAAA	88,7 %	Quiagen
Clathrin	AGCCTGAAAGTATCCGTAAAT	94,2 %	Quiagen
Giantin	AACTTCATGCGAAGGCCAAAT	90,4 %	Quiagen
Giantin[-2]3'UTR	TTGGAATTCTCTCACCTCTAA	73,0 %	Quiagen
GOLGA2[-1] (GM-130)	CAGGCTGGAGTTATACAAGAA	77,9 %	Quiagen
GOLGA3[-1] (Golgin160)	CTGGCCGATTACAGAACTGAA	65,3 %	Quiagen
GOLGA5[-1] (Golgin84)	CTGAGTTTAGTGGTCCTAATA	85,7 %	Quiagen
GOLGA5[-1r] (Golgin84)	CTGAGTTTAGTGGTCCTAATA	77,5 %	Quiagen
GOLGA5[-3] (Golgin84)	CACGACCAACCATATGGCAAA	88,5 %	Quiagen
GOLGA5[-4] (Golgin84)	AAGAAGATCTTTATCGAACAA	85,5 %	Quiagen
GOSR1[-1]	CACTGTGGTATTTATAGTATT	52,3 %	Quiagen
Luciferase	AACTTACGCTGAGTACTTCGA		Quiagen
Mss4	CTTCTCTCGCCGACAGCTTTT	87,1 %	Quiagen
NSF[-1]	AAGGAATGCAATAAAGAGTAA	62,6 %	Quiagen
NSF[-2]	CAGGTACACATTTACACTGAA	76,9 %	Quiagen
NSF[-4]	CACATAAAGGCCAGTACTAAA	72,9 %	Quiagen
p115 (VDP)	AACCCACCAAGACCGGCAATT	89,2 %	Quiagen
p115 (VDP)[-r1]	AACCCACCAAGACCGGCAATT	86,2 %	Quiagen
Rab11A [-1]	AAGAGTAATCTCCTGTCTCGA	95,0 %	Quiagen
Rab1A [-1]	GTCCAGCATGAATCCCGAATA	90,2 %	Quiagen
Rab1A[-2]	TAGGTTTGCAGATGATACATA	89,5 %	Quiagen
Sar1p	AAGCACGTCGCGTTTGGAAAA	94,1 %	Quiagen
Sec22b[-1]	TAGGACATTCTCAAATTTCAA	52,1 %	Quiagen
Sec22b[-2]	CCACAATTTGCTAACATTTAA	63,7 %	Quiagen
Sec22b[-3]	ACCTCATTTAATGAAGCTTAA	84,4 %	Quiagen
Stx5A[-1]	AAGTCCCTCTTTGATGATAAA	76,1 %	Quiagen
Stx5A[-1r1]	AAGTCCCTCTTTGATGATAAA	84,5 %	Quiagen
VAMP4[-1]	CAGGACAAATCAGAAAGCTTA	75,8 %	Quiagen
Ykt6[-1]	CTGGATAAAGTTGTCTTGAAA	81,1 %	Quiagen

xyN19 marks the bases complementary to the 3´ overhang of the anti-sense strand.

Listed siRNAs were obtained from Quiagen by the MPIIB si-RNA Core-Facility. Here also the cited knock-down was checked.

2. 8 Kits

Name	Supplier	Cat No. #
ECL Detection solution	PerkinElmer	# 2755
PhosphoScan®Kit (P-Tyr-100)	Invitrogen	# 9700
RNAiFect transfection reagent (Buffer EC-R)	Qiagen	# 301605
BCA protein assay	Thermo Fisher Scientific	# 23221

Methods

2. 9 Eukaryotic cells

2. 9. 1 Cultivation of human epithelial cells

Adherent cells were grown in 75 cm^2-flasks containing the respective culture medium at 37°C in a water-saturated, 5 % CO_2-containing atmosphere. To split and passage the cells medium of sub-confluent cells was aspirated; cells were washed with PBS and incubated with 1 ml Trypsin/ EDTA at 37°C for 5-10 minutes. After cell dissolution, 11 ml of fresh medium was added and a fraction depending on the desired dilution was transferred to a new flask. Medium was added to reach a final volume of 12 ml and the cells were again grown under the aforementioned conditions. To avoid passage-dependant secondary effects cell lines were renewed after the 20^{th} passage.
To seed a distinct amount of cells (e.g. for experiments in multi-well plates), a small volume (30 µl) of cell suspension was transferred to a Neubauer chamber and all cells within the four big squares were counted, with one square containing 0.1 µl. The concentration of cells/ ml was calculated as follows:

Cells/ ml = counted cells/ 0.4 µl x 1000 µl/ ml

Cells were then seeded in a volume according to the desired dilution and applied to the cell culture dish.

Cell culture vessel	Growth area (cm^2)	Number of cells
Multi-well plates		
24-well	2	2.5×10^5
12-well	4	5×10^5
6-well	9,5	1×10^6
Dishes		
100 mm	56	7×10^6
Flasks		
250-300 ml	75	1×10^7
650-750 ml	162-175	2×10^7

2. 9. 2 Cryo-conservation of eukaryotic cells

To conserve eukaryotic cells at least 1 x 10^6 cells were trypsinised and centrifuged at 800 g in a table top centrifuge. The supernatant was discarded and the cell pellet suspended in 10 % (v/ v) DMSO. The mixture was then transferred to pre-cooled cryo-tubes (Nunc) and stored over night at -80°C. The stock was kept in cryo-tubes in liquid nitrogen.

2. 9. 3 Defreeze and re-cultivation of eukaryotic cells

Freezed cells were thawed in a 37°C water-bath. Subsequently they were centrifuged in a table top centrifuge at 800 g, the supernatant discarded and the pellet resuspended in the respective cell culture medium. The cells were transferred into a 75 cm^2 cell culture flask and incubated over night at 37°C and 5 % CO_2. The next day the medium was discarded and fresh medium was added.

2. 9. 4 Amoeba cultivation

Acanthamoeba castellanii were cultivated in 150 cm^2 cell culture flasks at room temperature. To passage and split, the culture was washed with amoeba medium (table below) and the cells dissolved by gentle rapping. An appropriate amount of the suspended amoeba was then transferred to a new culture flask into fresh medium.

Medium	Amount	Composition
PYG 712-medium	10 g	Proteose-Peptone
	0,5 g	Yeast-extract
	0,5 g	Na- Citrate add 500 ml H_2O_{dd}, sterile filtration
	25 ml	2 M Glucose (autoclaved)
	4 ml	0,05 M $CaCl_2$ (autoclaved)
	5 ml	0,25 M KH_2PO_4 (autoclaved)
	5 ml	0,4 M $MgSO_4$ (autoclaved)
	5 ml	0,25 M $Na_2 HPO_4$ (autoclaved)
	5 ml	0,005 M Fe $(NH_4)_2$ $(SO_4)_2$ (autoclaved)
PYG 712-infection-medium	10 g	Proteose-Peptone
	0,5 g	Yeast-extract
	0,5 g	Na- Citrate add 500 ml H_2O_{dd}, sterile filtration
	25 ml	2 M Glucose (autoclaved)
	4 ml	0,05 M $CaCl_2$ (autoclaved)
	5 ml	0,25 M KH_2PO_4 (autoclaved)
	5 ml	0,4 M $MgSO_4$ (autoclaved)
	5 ml	0,25 M $Na_2 HPO_4$ (autoclaved)
	5 ml	0,005 M Fe $(NH_4)_2$ $(SO_4)_2$ (autoclaved)

(Steinert, M., M. Ott, P. C. Lück, E. Tannich, and J. Hacker. 1994. Studies on the uptake and intracellular replication of Legionella pneumophila in protozoa and in macrophage-like cells. FEMS Microbiol. Ecol. 15:299-308)
To prepare an amoeba culture for infection the cells were washed and split one day prior and seeded into well plates. They adhered over night and then were ready for infection.

2. 10 Bacteria

2. 10. 1 Growth of *L. pneumophila*

L. pneumophila cultures were grown on BCYE-agar plates (with or without antibiotics) for 24-48 h at 37°C in a water-saturated, 5 % CO_2-containing atmosphere. To reach a definitive amount of bacteria, these were taken off the plate using a cotton swab and were suspended in AYE broth or respective cell culture medium. The optical density should between 0.1 and 0.4 (OD600) was ascertained to equal $1x10^8$ bacteria/ ml. To stock the bacteria 700 µl of bacterial suspension were mixed with 300 µl of glycerol (86 %) and stored at -80°C.

Medium	**Amount (1l)**	**Composition**
AYE-broth (pH 6.9)	10,0 g	Yeast extract
	10,0 g	ACES supplement
	adjust pH to 6,9	
	2,0 g	Active charcoal
	autoclave	
	add sterile filtered:	
	0,25 g (in 10 ml) Fe (III) pyrophosphate	
	0,4 g (in 10 ml) L –Cysteine	
BCYE (pH 6.9)-agar plates	10,0 g	Yeast extract
	10,0 g	ACES supplement
	adjust pH to 6,9	
	2,0 g	Active charcoal
	15,0 g	Agar
	autoclave	
	add sterile filtered	
	0,25 g (in 10 ml) Fe (III) pyrophosphate	
	0,4 g (in 10 ml) L –Cysteine	

2. 10. 1. 1 *Legionella* preparation for infection

Legionella stocks are inoculated on BCYE-agar plates (if needed the plates contain antibiotics) 3 days prior to infection at 37°C und 5 % CO_2. For infection the bacteria are taken from the plate using a cotton swab soaked in the respective cell medium and suspended in the medium. The optical density of the bacteria suspension is measured and adapted to OD_{600} of 0, 3 having been established to come up to $1x10^8$ bacteria/ ml. From this stock solution dilutions can be made to answer different MOI´s (0.1-100) corresponding to the host cell amount.

2. 11 *L. pneumophila* infection of eukaryotic cells

2. 11. 1 Gentamicin-protection-Assay

To quantify intracellular growth in different host cell systems it is common to employ a Gentamicin-protection-assay. For this bacteria grow within a defined time lap (0 h, 7 h, 24 h and 48 h) inside the host cell after which the cells are lysed and the bacterial amount is estimated by CFU (colony forming units).
The host cells were treated according to cell cultural indications (2. 9. 1). Seeded one day prior to infection into 6-, 12-, or 24-well plates or in 75 cm^2 flasks they were 90 % confluent the day of infection. For infection the epithelial host cells were first starved of FCS for 30 min. Then the prepared bacterial suspension with the MOI corresponding to the amount of cells is co cultivated with the cells. In this study the epithelial cells were infected with MOIs of 10 or 100 corresponding to the experimental design. The well plates were then centrifuged at 800 rpm at 37°C for 5 minutes to enhance infection and then incubated at 37°C and 5 % CO_2. After 1 h Gentamicin at a concentration of 100 µg/ ml was added and incubated with the cells for 1 h to kill extracellular bacteria. The cells were then washed thoroughly with medium, fresh medium was added and the incubation proceeded. After the desired replication time (3 h, 6 h, 24 h, 48 h, 72 h) the cells were lysed using 0, 1 % saponin and plated on BCYE-agar plates using different dilutions. The first time point always was taken 3 h after adding the bacteria to the cells. Later time points were treated accordingly.

Solution	Amount	Composition
Saponin solution	1 % (w/ v)	Saponin in H_2O_{dd}, sterile filtrated
Gentamicin solution	50 µg/ ml	Gentamicin in RPMI 1640

2. 11. 2 *L. pneumophila* infection of amoeba

Amoebae were infected according to the cell-infection protocol described in 2. 11. 1 with MOI of 1 and 10 at 37°C and without centrifugation, as well as without Gentamicin treatment.

2. 12 Nucleic acid methods

2. 12. 1 siRNA transfection of eukaryotic cells

In molecular biology RNAi is used to specifically down regulate gene expression. It is based on the capability of RNA oligonucleotides with a complementary sequence to interfere specifically with messenger RNA (mRNA) translation of the targeted gene by either blocking the proceeding ribosome on the mRNA molecule or by initiating its degradation. The latter takes place in eukaryotic cells initiated by the ATP-dependent cleavage of double-stranded RNA (dsRNA) molecules through the RNase III family member dicer and results in smaller oligonucleotides, the so-called siRNAs (small interfering RNAs). These molecules with 20-25 nucleotides have a phosphorylated 5'-end and a none-phosphorylated 3'-end. One siRNA molecule associates with the argonaute endonuclease to form the RNA induced silencing complex (RISC) that separates the RNA strands from each other. The guiding strand remains in the complex targeting the complementary mRNA to allow its degradation by the argonaute protein Ago2 (Dykxhoorn *et al.* 2003)

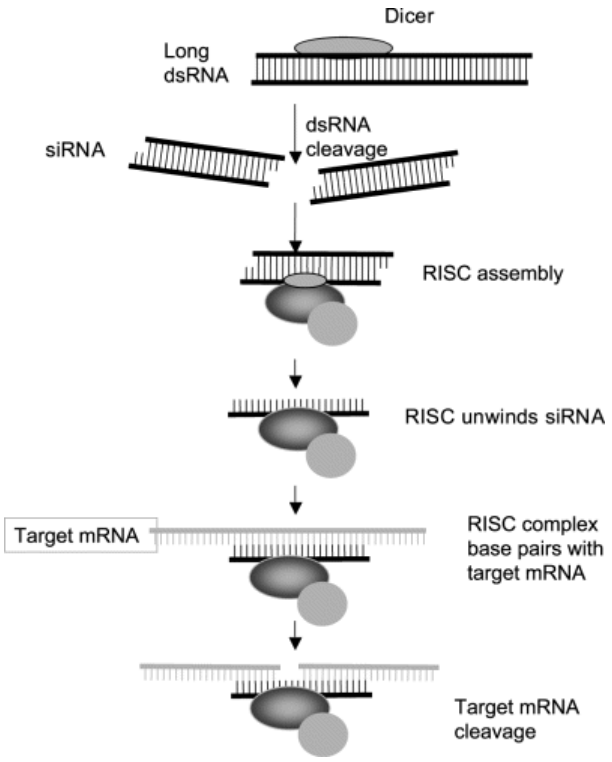

Fig. 2. 1. A generally accepted model of the RNA interference (RNAi) mechanism.
Small interfering RNA (siRNA; 21–23 bp fragments) with 2 nucleotide 3' overhangs is introduced into the cell. Followed by cleavage via the RNA-induced silencing complex (RISC) that loads and unwinds the siRNA, and binds to the complementary target mRNA. This subsequently is cleaved by the RISC. Following cleavage, the RISC complex disassembles and is ready to load another siRNA for cleavage of additional mRNA (modified after Karpala *et al.* 2005).

In this work lyophilised oligonucleotides were suspended in siRNA Suspension Buffer (Qiagen) to obtain a 20 µM solution. The suspension was portioned in 5 µl aliquots and stored at -20°C. For siRNA transfection adherent cells were grown to 50 % confluence in 24-well plates (1x 10^5 cells). 1 µg of the desired iRNA was diluted in 96 µl RNAiFect ECR-Buffer (Qiagen). Then 6 µl of RNAiFect reagent was added, mixed and left for incubation for 15 minutes. During this time liposomes were building around the siRNAs to later facilitate entry into the cells. After incubation the cell medium was aspirated and replaced by 300 µl of FCS free medium. Then the RNAi-mix was added drop by drop while the plate shook gently. Transfection mixture was incubated with the cells for 3 days and protein levels of cell lysates were validated by Western blot.

2. 13 Protein biochemical methods

2. 13. 1 Detection of protein amount (BCA)

The BCA Protein Assay combines the reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{1+}) by bicinchonic acid. Single amino acids and dipeptides do not give the biuret reaction, but tripeptides and larger polypeptides or proteins will react to produce a light blue to violet complex that absorbs light at 540 nm. One cupric ion forms a colored coordination complex with four to six nearby peptide bonds. The intensity of the color produced is proportional to the number of peptide bonds participating in the reaction.

The sample preparation for a BCA protein assay started with washing of the cells with ice-cold sterile PBS. Fresh PBS was added and the cells were scraped and collected in a 1, 5 ml tube and stored on ice. The samples were then centrifuged at 4°C at 14000 rpm for 5 minutes. The supernatant was discarded and the samples could be store at -20°C or, the experiment proceeded. The pellet was resuspended in 100µl Complete and incubated for 30 minutes in ice, vortexing of the samples every 10 minutes. Subsequent centrifugation for 30 minutes at 14000 rpm at 4°C should have left solved proteins in the supernatant. This was transferred to a fresh tube. BSA standards were prepared in a row of dilution (2 mg/ ml; 1 mg/ ml; 0, 5 mg/ ml; 0, 25 mg/ ml; 0, 125 mg/ ml). These were filled into the valves of an ELISA plate as well as a blank sample of RIPA buffer and the samples. Then the reaction solutions A and B were mixed according to the protocol (50: 1) and 200 µl were added onto the samples and the standard. The plate was incubated for 30 minutes at 37°C and then measured in an ELISA reader at 560 nm. The wavelength corresponded to the amount of protein given in the sample.

Reagent	Amount	Component
RIPA (store at 4°C)	50 mM	Tris/ HCl (pH 7,5)
	150 nM	NaCl
	1 %	NP-40
	1 %	TritonX 100
	0,1 %	SDS
Complete	25x stock	in H_20_{dd}

2. 13. 2 Discontinuous SDS polyacrylamide gel electrophoresis (SDS-PAGE)

To detect proteins of different sizes this method of a discontinuous SDS-polyacrylamide-gel-electrophoresis was employed according to Laemmli (1970). In the first step SDS denatures the proteins. SDS-anions bind to amino acid residues in constant weight relations. The negative loading of the resulting complex of SDS and the denatured protein is proportional to its weight. The negatively charged complex proceeds towards the anode where the smaller proteins have a higher electrophoretic motility and travel faster through the polyacrylamide gel. The molecular masses

detected then are deduced from the protein monomers under reduced terms.

Solution	**Amount**	**Composition**
Acryl amide stock solution	30 %	(30 %, w/ v): Acryl amide
	0.8 %	N, N-Methylen-bis-Acrylamid
10x SDS Separation gel-buffer	1.5 M	Tris/ HCl
(pH 8.8)	0.4 % (w/ v)	Sodiumdodecylsulfate (SDS)
10x SDS Collecting gel-buffer	0.5 M	Tris/ HCl
(pH 6.8)	0.4 % (w/ v)	Sodiumdodecylsulfate (SDS)
10x SDS Running buffer	0.5 M	Tris/ HCl
	3.84 M	Glycine
	0.1 % (w/ v)	SDS
5x Sample buffer	5 %	SDS
	0.5 %	Bromophenol blue
	0.25 M	Tris/ HCl pH 6.8
	10 mM	EDTA
	1 %	β-Mercaptoethanol
	50 %	Glycerol
Isopropanol (100 %)		
TEMED		
APS (10 %)		

The electrophoresis apparatus was prepared and assembled according to the manufacturer's instructions. The glass plates were cleaned with 100 % EtOH. The separation gel was mixed amounting different percentages according to the protein sizes that were to be detected.

15 % Gel	10 % Gel	7,5 % Gel	5 % Gel
12-43 kDa	16-68 kDa	36-94 kDa	57-212 kDa

The freshly prepared separation gel was filled in between the two glass plates of the assembled chamber up to 1, 5- 3 cm below the brink. Isopropanol was added to obtain a clear cut. After complete polymerization of the gel Isopropanol was removed and the collecting gel solution was added, topped by the comb. The polymerised gel between the class plates was then assembled into the electrophoresis chamber and the reservoirs filled with SDS running buffer. The sample slots were rinsed to remove gel residues before adding the samples. After run for 10 minutes at 70V until the samples had passed the collecting gel, then turn to 150V till the end. After approximately 1 h, when the bromphenol leaks out at the bottom, the run was terminated. Following this the gel was stained using Coomassie-Blue or further treated for Western blotting.

Separating gel					**Collecting gel**
Concentration of acrylamide	5%	10%	12%	15%	
30% Polyacrylamide	1,75 ml	3,3 ml	3,9 ml	4,75 ml	1 ml
Separating-gel buffer	3,76 ml	3,76 ml	3,76 ml	3,76 ml	500 µl
H_2O_{dd}	4,39 ml	2,76 ml	2,46 ml	1,31 ml	2,42 ml
10% SDS	100 µl	100 µl	100 µl	100 µl	40 µl
10% APS	80 µl	80 µl	80 µl	80 µl	40 µl
TEMED	8 µl	8 µl	8 µl	8 µl	5 µl

Coomassie staining

To stain and fix polyacrylamide gels, these were gently shaken in Coomassie-blue at 40 rpm for 1-2 h or over night at room temperature. To de-stain they were shaken in de-stain-solution that had to be refreshed several times. After successful de-stain of the background the gel was be stored in H_2O or dried in a gel-dryer for 2 h at 70°C.

Solution	**Amount**	**Composition**
Coomassie staining solution	0,25 % (w/ v)	Coomassie Brilliant Blue R250
	50 %	MeOH
	10 %	glacial acetic acid
De-staining solution	10 %	EtOH
	10 %	glacial acetic acid

2. 13. 3 Immunoblot (Western blot)

Via the Western-Blot technique proteins can be transferred PVDF-membrane by electrophoresis (Towbin 1979) so they can be analysed. Proteins are blotted to a PVDF membrane either by the semi-dry or wet blot technique.

In both cases, PVDF membrane of a size corresponding to the applied gel was activated by incubation in MeOH for 15 seconds. The membrane then was washed with water for 3 minutes. For semi-dry blotting, two layers of wetted Whatman-paper were placed on the platinum-covered anode of the blotting chamber (Biometra), followed by the membrane, the gel and again two layers of Whatman-paper. The chamber was closed with the anode lid and a current of 50 mA/ blot was adjusted and run for 90 minutes / membrane.

Transfer by wet blot was assembled in a blot column on the black part of the gel holder cassette, with one fibre pad on each side. Cassettes were closed and inserted into the electrode assembly that was then placed in the buffer tank, the buffer being constantly stirred at 4°C. A current of 250 mA was applied for a 3 h of transfer. To run transfer over night, the current was adjusted to 150 mA.

Buffer	Amount	Composition
Running buffer	25 mM	Tris/ HCl pH 8.3
	192 mM	Glycine
	0,1 % (w/ v)	SDS
Transfer buffer (Wet blot)	25 mM	Tris
	190 mM	Glycine
	20 %	MeOH
Transfer buffer (Semi dry)	48 mM	Tris
	39 mM	Glycine
	0,035 mM	SDS
	20 % (v/ v)	MeOH

2. 13. 4 Detection of antigen-antibody reaction

To detect specific proteins on the membrane, antibodies for a single polypeptide are used. An antibody solution is incubated with the membrane. A secondary antibody recognizing the F_c portion of the first antibody after being conjugated with horse-radish-peroxidase again, detects this antibody. A peroxidase substrate solution leads to a chemo luminescent signal detectable by photo films.

To inhibit unspecific antibody binding the membrane firstly was blocked by incubation in blocking buffer for 1 h at room temperature. Then the membrane was washed (5x 5 minutes) with TBS-T while gently shaking and then incubated with the primary antibody solution (dilution depending on the antibody 1:500 - 1:5000) in a 50 ml Falcon tube for 1 h. The membrane was washed again (4x 5 minutes) and the secondary antibody (1: 3000) solution is applied for 50 minutes. Solution was discarded and the membrane subjected to a final washing step (3x 5 minutes) before visualization by means of enhanced chemo-luminescence (ECL) reaction.

Buffer	Amount	Composition
Blocking solution	10 %	non-fat dry milk
	3 % (w/ v)	BSA in TBS-T
Wash Buffer (TBS-T)	10 mM	Tris/ HCl pH 7.5
	100 mM	NaCl
	0,1 %	Tween-20

2. 13. 5 Chemo luminescence (ECL) detection

To detect peroxidase linked antibodies the membrane is incubated with a detection solution. The peroxydase catalyses the reduction from O_2 to H_2O_2 that oxidizes the Luminol this again produces blue light.

The solution was mixed 1:1 detection solution A (hydrogen peroxide) and B (Luminol) for 1 minute. It was then decanted and the membrane was enveloped into a foil and placed into the development

cassette. In the dark room a blue-light screen was applied and developed. The film was developed in a developer machine.

2. 13. 6 Membrane stripping

One membrane already coated with antibodies cannot be used again for other reactions. For this the bound antibody needs to be detached from the membrane and the membrane subsequently has to be blocked afresh.

To strip a membrane it was incubated for 30 minutes at 50°C while shaking in strip buffer, followed by rigorous washing (2x 10 minutes in TBS-T) and blocked for another 1h.

Buffer	Amount	Composition
Stripping Buffer	62.5 mM	Tris/ HCl pH 6.7
	100 mM	β-Mercaptoethanol
	2 %	SDS
Blocking Buffer	10 %	Non-fat dry milk
	3 % (w/ v)	BSA in TBS-T

2. 13. 7 Protein co-immuno-precipitation (P-Tyr Kit)

The PhosphoScan Kit from Cell Signalling Technology allows for the purification and identification of tyrosine phosphorylation sites in cellular proteins when coupled with LC tandem mass spectrometry. The assay is based on the specific enrichment of phospho-tyrosine –containing peptides using an antibody against phospho-tyrosine. Cells are lysed in a urea-containing buffer, and cellular proteins are digested by protease and fractionated by reversed-phase solid-phase extraction. Peptides are then subjected to immuno-affinity purification using Phospho-tyrosine mouse antibody (P-Tyr-100) #9411 coupled to protein G-Agars beads. Over night incubation ensures high-affinity binding of phospho-tyrosine-containing peptides to P-Tyr-100 beads. Unbound peptides are removed through washing, and phospho-tyrosine containing peptides are eluted with dilute acid. Reversed phase chromatography is performed on micro tips to separate phospho-peptides from antibody and to concentrate them for LC tandem mass spectrometry.

2x 10^8 cells (approximately corresponds to 10x 150 mm culture dishes grown to 70-80 % confluence) were either infected with *L. pneumophila* or remained uninfected, washed with cold (4°C) PBS and 10 ml of chilled lysis buffer/ 10 plates was added and the cells scraped. The lysate was then sonicated and cleared by centrifugation. The extracted protein was carboxymethylated by adding 1/ volume of 45 mM DTT to the earned cell supernatant. This was mixed and incubated for 20 min at 60°C. The solution then was cooled to room temperature and 100 mM iodacetamide was added at an equal volume as the DTT solution, mixed well and incubated for 15 min at room temperature in the dark. The following trypsine-digestion was carried out in a 4-fold dilution to a final concentration of 2 M urea, and 20 mM HEPES buffer, pH 8.0 and mixed. A 50 ml aliquot of this solution was used for SDS

PAGE analysis. The sample was then digested by 1/ 100 volume of a 1 mg/ ml trypsine-TPCK solution over night.

2. 13. 8 Molecular mass determinations by MALDI mass spectrometry

This method is used to directly identify proteins contained in mixtures by micro column reversed-phase liquid chromatography electro spray ionization tandem mass spectrometry (LC/MS/MS). A mixture of proteins is digested with a proteolytic enzyme to produce a large collection of peptides. The complex peptide mixture is then separated on-line with a tandem mass spectrometer, acquiring large numbers of tandem mass spectra. With the help of a protein database it is possible to identify the proteins present (McCormack 1997). Monica Schmitt of the protein facility of P. Jungblut from the proteomics-core-facility carried out LC tandem mass spectrometry.

2. 14 Fluorescence based methods and microscopic studies

2. 14. 1 Immunofluorescence staining

TO analyse the exact localisation of distinct proteins within a cell immunofluorescence is employed. Here for proteins are marked using specific anti-bodies and these again are detected via fluorescently labelled secondary antibodies. The emission of the fluorochrome coupled to the secondary antibody is made visible via a confocal microscope enabling further analysis. Confocal microscopy delivers pictures of several layers of the cell so that exact localisation of the protein within the cell is possible.

Cells were grown on cover slips in a 24-well multi-well plate. They were washed with PBS and fixed with 4 % PFA for 30 minutes at RT. The cells were then again washed twice with PBS and auto fluorescence was quenched with 0, 1 glycine solution for 15 minutes at room temperature. The washing was again repeated with PBS. The slips could then be either stored at 4°C for later proceeding or stained immediately. The staining procedure started with permeabilisation with 0, 1 % TritonX-100/ PBS for 20 minutes at RT, washing twice with PBS for 5 minutes and blocking with 1 % BSA in 0, 05 % Tween20/ PBS for 20 minutes. The cover slips were then incubated on a 50 µl drop of primary antibody solution (1: 100 in blocking solution) placed on a multi-well plate lid covered with parafilm for 1 h to allow binding. There after the cover slips were washed in a multi-well plate three times with PBS. Secondary antibody binding was performed according to primary antibody binding procedure, but with a 1:150 dilution. For actin staining, phaloidin coupled with either Alexa Fluor 546 or 647 was added to the secondary antibody solution in a 1: 100 dilution. After washing three times with PBS, cover slips were rinsed with H_2O_{dd} and mounted on a glass slide with Mowiol.

Solution	Amount	Composition
4 % PFA pH 7.4	8 g	PFA
(store at -20°C)	100 ml	H_2O_{dd}
	heat up to to 65°C	
	add 1 M NaOH until solution is clear	
	20 ml	10x PBS
	adjust pH to 7.4	
	add H_2O_{dd} to final volume of 200 ml	
Permeabilisation-solution	0, 1 %	TritonX-100 in PBS
PBS	15 mM	NaCl
(Phosphate buffered saline)	0, 84 mM	Na_2HPO_4
	0, 18 mM	NaH_2PO_4 (pH 7.4)
Blocking-buffer	1 %	BSA
	0, 05 %	Tween20 in PBS
Mowiol	2, 4 g	Mowiol 4-88
(store at 4°C)	7 g	Glycerol
	6 ml	H_2O_{dd}
	stir at RT for 2 h	
	0, 2 M	Tris/ HCL (pH 8.5)
	stir at 50°C for 1h	

2. 14. 2 Confocal microscopy

For the microscopy studies a Leica TCS SP microscope was used, equipped with an argon/ krypton laser. Specimens were examined at a 630x magnification using immersion oil. Due to the transmission filter of the microscope, three different excitation wave lengths could be applied at the same time allowing the parallel observation of three differently fluorescence labelled proteins/ structures.

2. 14. 3 NF-κB activation readout-system

To investigate NF-κB activation upon *L. pneumophila* infection, a clonal reporter cell line was employed (A549 SIB01), expressing p65 (RelA) as a GFP-fusion protein (Bartfeld *et al.* 2009). In non-activated cells p65 is located to the cytoplasm, bound to its inhibitor IκBα. Stimulation via for example bacterial effectors activates the IκB-kinase of the (IKK)-complex, phosphorylating IκBα, subsequently inducing proteasomal degradation of the p65 inhibitor IκBα. P65 now is released and translocates into the nucleus where it binds to DNA in the manner of a transcription factor inducing gene expression of several genes as for example Il-8 or Cox2. An automated fluorescence microscope (Scan^R; Olympus) visualized active and non-active cells in a 96-well-standard (Fig. 2. 2a). Active cells differed from inactive ones by nuclear localisation of the p65-GFP-fusion protein.

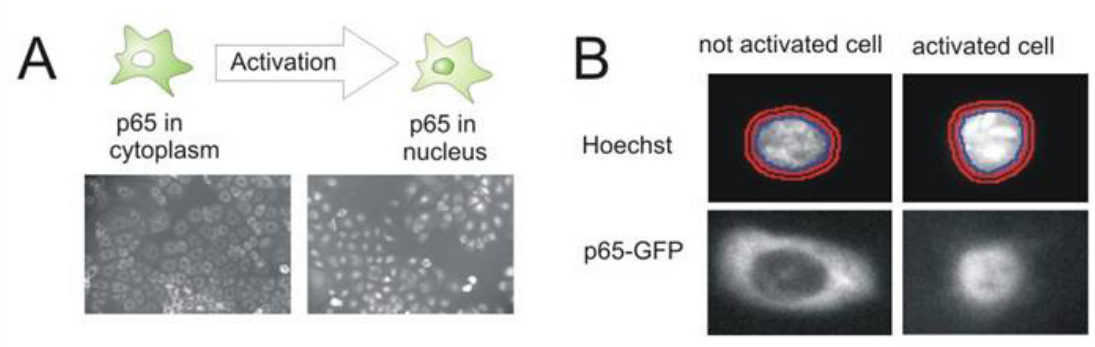

Fig. 2. 2a. Schematic over-view of the NF-κB read-out: p65-GFP reporter cells and automated microscopy.
A) The epithelial reporter cell line stably expresses the GFP fusion protein of the NF-κB subunit p65. After activation the p65-GFP translocates into the nucleus. B) Sub cellular areas defined for the translocation assay in the Scan^R Analysis software: nucleus (blue); cytosol (red). Cells were fixed and stained with Hoechst 33342. Depicted are one activated and one non-activated cell (Bartfeld *et al.* 2009)

Computational analysis enabled quantitative measurement by the definition of the ratio of activated to non-activated cells. To distinguish these cells, the cell nuclei were stained with Hoechst 33342 (1: 10000). The software was able to recognise and analyse each blue cell as a singular object. The captured cells were then divided into defined cell populations of active and inactive cells (Fig. 2. 2b). The Heywood Circularity Factor (HCF), defined by: particle diameter/ (2 x √ (π x particle expanse)) was used to select a cell population from a Dot plot diagram possessing normal circularity and size (region (R02)). From this one population two more were classified differing in the amount of nuclear to cytosolic GFP signal in reference to the cytosolic GFP-median. A ration higher than one classified for active cells, lower than one depicts inactive cells.

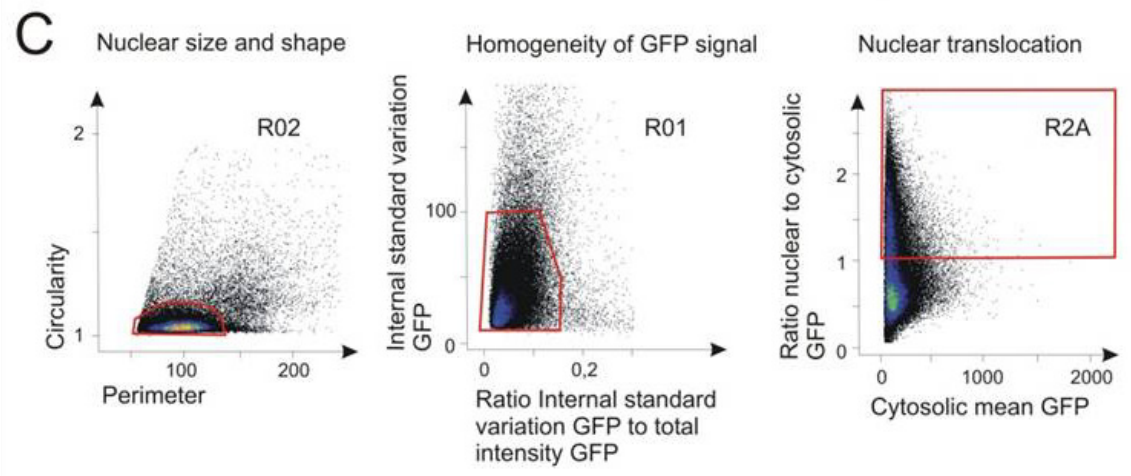

Fig. 2. 2b. Schematic over-view of the NF-κB read-out: p65-GFP reporter cells and automated microscopy.
C) Translocation assay using Scan^R Analysis. Cells are gated for circularity and size (region R02), intensity of GFP and standard variation of GFP intensity (region R01) and the ratio of nuclear to cytoplasmic GFP intensity (region R2A). Cells are named "active" when they are in gates R01, R02 and R2A (Bartfeld *et al.* 2009).

The receptor cell line responds to NF-κB stimuli in the same way as the parental line of wt cells. This was verified by infection of cells with the pathogen at indicated MOI and for the given time and detection of degradation of IκBα and actin control analysed by western blot. The automated read-out gives the same results as the western blot.

3 Results

3. 1 Establishment of optimal *L. pneumophila* infection model setting

To investigate where inside its host cell *L. pneumophila* specifically interacts with cellular trafficking components and to learn more about the differences in the pathways that are hijacked by this bacterium – in comparison to *Salmonella* or *Chlamydia* for example - it first was necessary to have a working *L. pneumophila* infection system. The *L. pneumophila* wild type strains used were Corby, JR32, Philadelphia01, the mutant strain was JR32*ΔdotA* (defective in the pathogens type IV secretion system). Since the infection assays for *Chlamydia* and for *Salmonella* were already established in HeLa (human cervix-carcinoma epithelial cells) model cells, as well as their transfection with siRNA, these cells were chosen. Although *L. pneumophila* mostly infect macrophages and do not efficiently infect epithelial cells, there is literature evidence for epithelial cell infection by *L. pneumophila* (Cattani *et al.* 1997, Kunishima *et al.* 2000). Since HeLa cells are a common model for epithelial cells and additionally show very efficient knock-down on protein level via siRNA, they were chosen for this work.

3. 1. 1 Growth of different *L. pneumophila* wild type strains in HeLa model cells

The first step was to find out, how different *L. pneumophila* wild type (wt) strains, as there were Corby, JR32 and Philadelphia01 and also the *ΔdotA* mutant in JR32 infected epithelial cells under different conditions. The conditions tested were: amount of bacteria given as multiplicity of infection (MOI) of 1, 10 and 100, the period of time (3 h, 24 h, 48 h and 72 h) and the cell type (HeLa and A549 cells) for ideal replication. A time course was designed and varying MOIs were compared in these two cell lines. For the later on planned “focussed-mini-screen” employing siRNA generated protein knock-down it was necessary to establish this model in epithelial cells.

Figure 3. 1a shows the growth of *L. pneumophila* wt strains Phil01 and JR32 in HeLa model cells, infected at an MOI of 10. Both strains showed replication of two-log phases after 24 h incubation. The number of intracellular *L. pneumophila* reached approximately 1 x 10^7 CFU/ ml for JR32 wt and 5x10^6 CFU/ ml for Phil01 wt at 24 h. After 48 h the amount of *L. pneumophila* decreased to 5 x10^5 CFU/ ml and 2 x10^5 CFU/ ml for both strains and after 72 h even further to 1x 10^5 CFU/ ml and 3x 10^4 CFU/ ml. This decrease in *L. pneumophila* detection after 48 h is due to the Gentamicin-containing medium into which the bacteria exited after disrupting their host cell after 24 h.

The growth shown below and in all of the following chapters are representatives of at least three different experiments each comprising triplets of one data point.

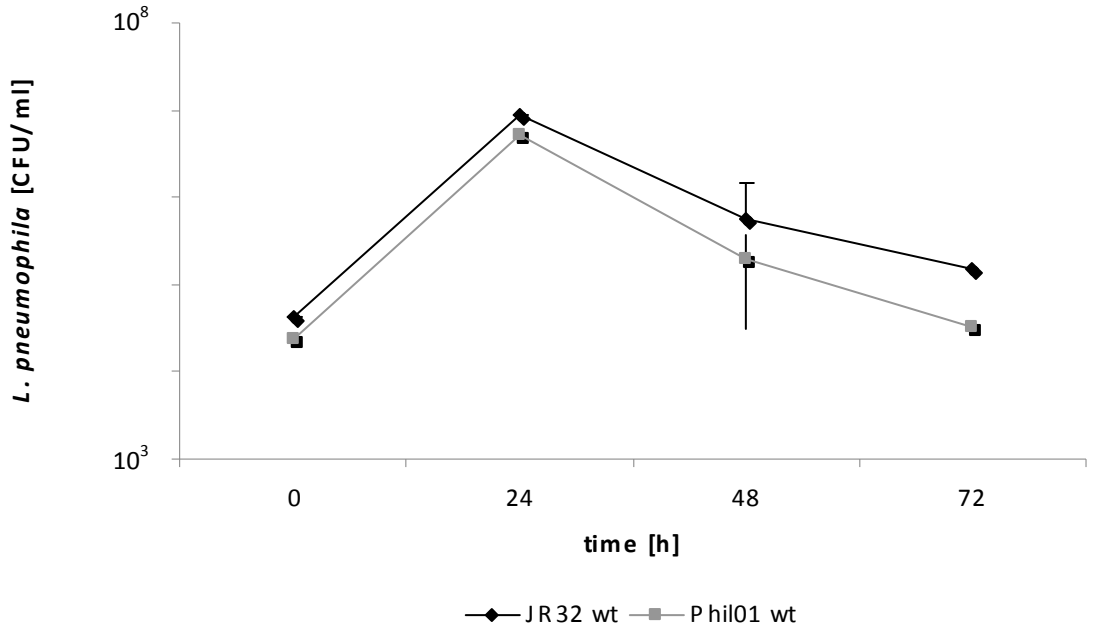

Fig. 3. 1a. Growth of *L. pneumophila* wt strains JR32 and Philadelphia01 in HeLa model cells.
HeLa model cells cultured in RPMI were infected at an MOI of 10. Infection was synchronised by centrifugation at 800 g for 5 min at 37 °C. After 90 min extracellular bacteria were removed by washing, remaining extracellular bacteria were killed by Gentamicin (100 µg/ ml for 1 h; 50µg/ ml until cell lysis). Cells were washed 3 times with medium to remove unbound bacteria and lysed with 1 % (w/ v) saponin (Sigma) and plated in serial dilutions on BCYE agar. Multiplication of *L. pneumophila* was assessed by CFU counting. Error bars = standard deviation of triplicates. Results are representative for three independent experiments. The blue line represents the growth of JR32 wt; the pink line shows the growth of Phil01 wt.

Other *L. pneumophila* wt strains were also compared at different multiplicities of infection for later time points. One example is an infection with Corby wt and Phil01 wt at an MOI of 100 for 48 h.

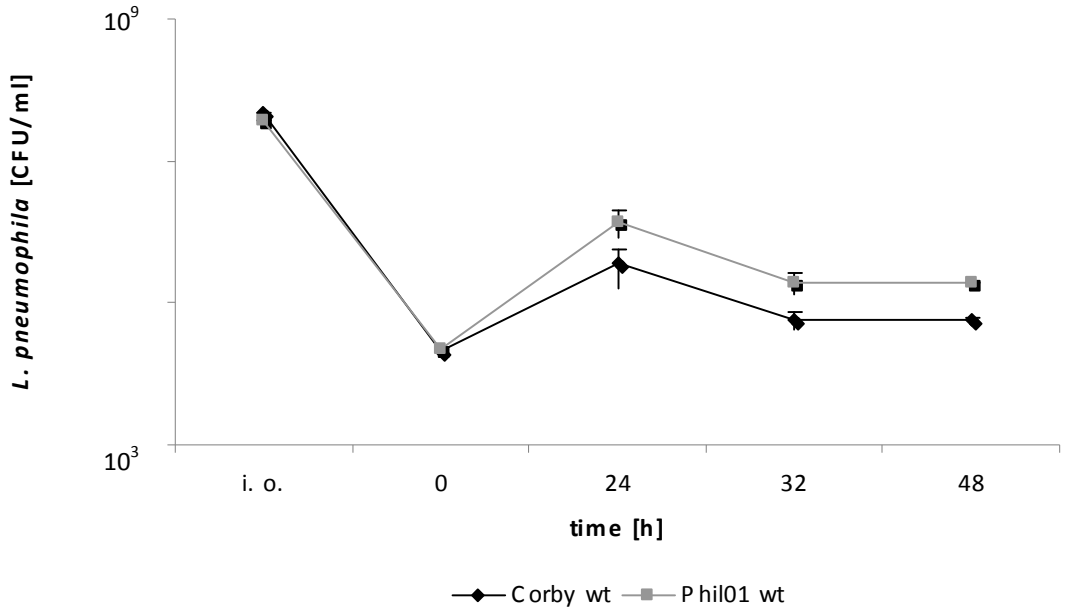

Fig. 3. 2. Replication of *L. pneumophila* wt strains Corby and Phil01 in HeLa model cells.
The host cells were infected using a MOI of 100. After phagocytosis and killing of extracellular bacteria by gentamicin (time point 0= phagocytosis rate), cells were incubated for further 24 h, 32 h and 48 h and the number of viable bacteria after intracellular multiplication was determined by disruption of the host cells and culture of the fluid on BCYE agar. Growth curves show the mean intracellular number of bacteria +/- S.D. of three independent experiments.

Figure 3. 2. shows how inefficient the *L. pneumophila* wt infection in epithelial cells was. From initially 5x 10^7 (MOI 100) only 3x 10^4 bacteria entered the cells. Also the Corby wt (1, 3 log phase) did not replicate to such high numbers as the Philadelphia01 (2 log phases) wt strain.

3. 1. 2 Growth of *L. pneumophila* Philadelphia01 wt at MOIs 10 and 100 in HeLa model cells

Since the given *L. pneumophila* strains did not replicate to high numbers in the given cell model, it was estimated which MOI of infection resulted in the highest amount of replicated bacteria, still being sensibly physiological. For this growth assay the Philadelphia01 wt strain was used at the MOIs 10 and 100 in HeLa model cells (Fig 3. 3). The cells were lysed at time points 3 h and 24 h and the replication stated after CFU count.

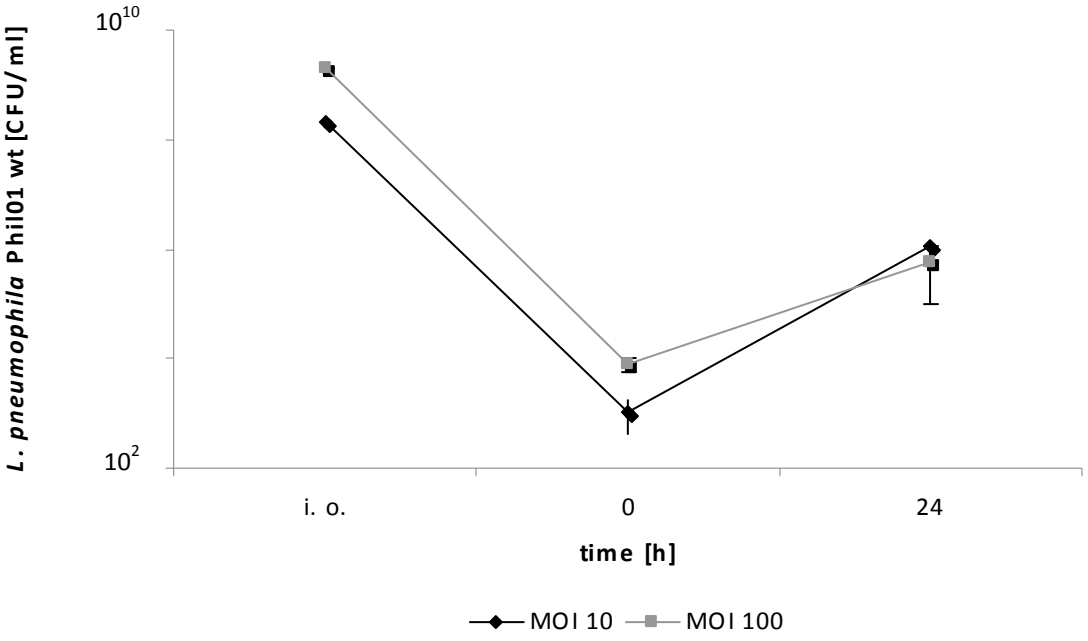

Fig. 3. 3. Replication of *L. pneumophila* Philadelphia01 wt strains in HeLa model cells at different MOIs.
The cells were infected with MOIs of 10 and 100. Amount of replicated bacteria was estimated by CFU count. Error bars = standard deviation of triplicates. Results are representative for three independent experiments.

Interestingly infection with a higher amount of bacteria resulted in higher infectivity but less replication. This might be true because of effective higher replication resulting in earlier disruption of the infected cells, and earlier egression of the bacteria into the Gentamicin-containing medium. The extracellular bacteria were killed so that less could be detected after the given time point of 24 h. Since siRNA down-regulation of cellular trafficking proteins is highest after 4 d (data shown below 3.2.1.1.) we decided to use the 24 h time point for read-out, to ensure maximal protein down-regulation throughout *L. pneumophila* replication.

3. 1. 3 Growth of *L. pneumophila* JR32 *ΔdotA* for 24 h in HeLa model cells

One important control for *L. pneumophila* growth experiments inside cells is to see whether bacteria lacking the type IV Dot/ Icm secretion system could replicate or not. The Dot/ Icm secretion system is important for translocation of bacterial effector molecules into the host cytosol enabling survival and replication. Δ*dot* mutants normally are replication deficient. The mutant given in this part is derived from the JR32 wt strain. In figures 3. 1a. and b. replication of the two wt strains JR32 and Philadelphia01 has already been compared, both strains giving comparable results. Philadelphia01 wt was a little less infective, than the JR32 wt. But both showed almost two log phase replication after 24 h.

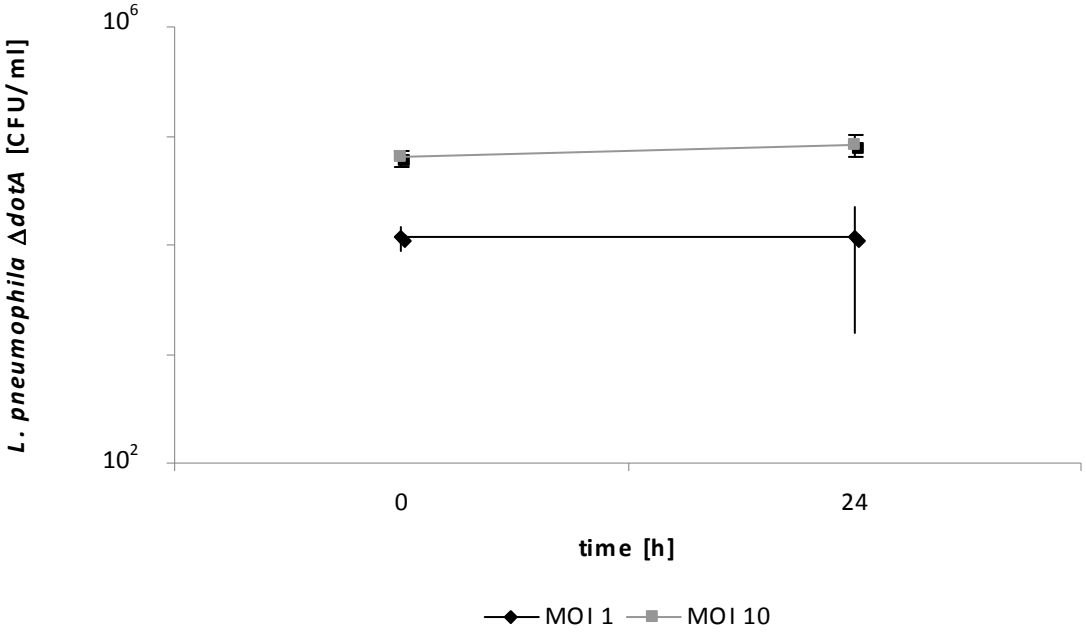

Fig. 3. 4. No replication of *L. pneumophila* JR32Δ*dotA* mutant in HeLa model cells at different MOIs.
Cells were infected with the mutant *L. pneumophila* at MOIs of 1 and 10. Amount of replicated bacteria was estimated by CFU count. Error bars = standard deviation of triplicates. Results are representative for three independent experiments.

The amount of Dot/ Icm defective *L. pneumophila* inside the host cell even decreased with time (error bars) (Fig 3.4). This might again be due to bacteria being killed after the cells had died and the *L. pneumophila* were exposed to the surrounding Gentamicin-containing medium.

3. 1. 4 Summary

From these first establishing experiments the conclusion can be drawn that *L. pneumophila* do not very efficiently infect epithelial cells (from 5x 10^7 bacteria only 3x 10^4 resided the cell 3 h after infection). The wt strains used all showed replication of at least 1 to 2 log phases. Even the MOI did not extremely alter replication. MOI of 100 resulted in the same degree of replication as MOI of 10. We chose to infect with MOI of 10 as being more physiological. From this we concluded that for the planned "focussed mini-screen" infection of the *L. pneumophila* wt strain Philadelphia01 at an MOI of 10 in HeLa model cells was most suitable.

3. 2 "Focussed mini-screen" set up

Aim of this part of the work was to find host cell trafficking proteins essential for *L. pneumophila* replication in human cells. The first step was, to establish transfection of the chosen HeLa model cells with siRNA to down-regulate distinct proteins. After the protein was successfully knocked-down, the cells were infected with the *L. pneumophila* wt strain Philadelphia01 at an MOI of 10. Later on the cells were lysed 3 h and 24 h post infection (p. i.), plated on BCYE-agar in serial dilutions and the CFUs counted after 72 h incubation at 37˚C. Via this assay the impact of the down-regulated host cell proteins on *L. pneumophila* replication within 24 h could be estimated and compared with non- or mock-transfected cells. Most of the host trafficking proteins chosen for the mini-screen had already been investigated concerning their impact on replication of *Chlamydia* and *Salmonella* (prior work at the MPIIB).

3. 2. 1 Transfection of siRNA in HeLa model cells

The method of siRNA transfection in epithelial cells had already been established at the MPIIB. Here also all of the siRNAs were designed and tested for their knock-down efficiency on mRNA level by RT-PCR (s.o. 2.7). Transfection of the desired siRNA was accomplished using the RNAIfect Kit from Qiagen. This method uses lipofectamine as a translocation reagent to enable the siRNA to enter the cell. Transfection of knock-down efficiency of several of the chosen trafficking proteins on protein level was tested to assure down regulation.

3. 2. 1. 1 Protein amount validation after siRNA knock-down (time course)

To estimate the amount of down-regulated protein in siRNA transfected cells, these were grown for 1-5 days and then analysed via antibody detection. The protein amount in the cells was measured using the BCA kit and after setting up an even amount of whole protein, SDS-PAGE electrophoresis was run und the proteins subsequently blotted to a membrane. Via specific antibody detection the amount of protein in transfected and un-transfected cells was made visible using the ECL-detection kit. One example of such a validation is depicted in Fig. 3. 5. below.

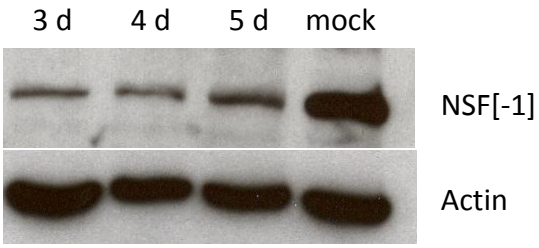

Fig. 3. 5. NSF[-1] protein knock-down validation after 3, 4 and 5 days.
The transfected cells were harvested 3, 4 and 5 days after transfection on ice and their total protein amount estimated by BCA. The total protein was separated via SDS-PAGE and blotted to a nitrocellulose membrane. Mock shows the protein amount of NSF in cells only treated with RNAIfect buffer and reagent after 4 days. NSF (70 kDa) (upper row) and actin (43 kDa) (lower row) were detected by specific antibodies

For trafficking proteins with a high turnover such as NSF, the knock-down was most efficient after 4 d. In all subsequent mini-screens at least one protein was checked for down-regulation on protein level by Western Blot analysis.

3. 2. 1. 2 Protein amount validation after siRNA knock-down (different siRNA sequences)

To verify given hits in the subsequent "mini-screen" these were further tested using different siRNA sequences targeting the same protein but with different RNA sequences. This was only done when down-regulation of one protein seemed to have an impact on *L. pneumophila* replication. I used varying sequences for NSF, Rab1, Stx5, CASP, Golgin84, Arf1, Giantin, p115 and Sec22B. Below is shown an example of three different siRNAs against NSF sequence (Fig. 3. 6).

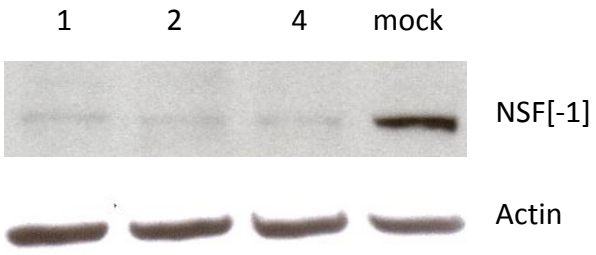

Fig. 3. 6. NSF[-1] protein knock-down validation for different siRNA sequences (1, 2 and 4).
HeLa model cells were transfected with three different siRNAs against NSF. After 4 d the cells were harvested and their protein amount measured via BCA. The total protein was separated via SDS-PAGE and blotted to a nitrocellulose membrane. NSF (70 kDa) and Actin (43 kDa) were detected via antibodies.

The siRNAs acquired from the core facility were all prior validated for their mRNA regulation via RT-PCR, where they had to give at least 60 % knock-down. Subsequently we tested protein knock-down for only one protein per screen set (7 proteins/ set), to verify successful transfection.

3. 2. 2 Target proteins

For this study we chose to investigate host cell proteins from the trafficking pathway: ER to Golgi apparatus, *intra*-Golgi apparatus, vesicular coat proteins, SNAREs and GDP´s (see tables below). Most of these had already been tested for their impact on *Chlamydia* or *Salmonella* replication (prior in the MPIIB).

Golgi apparatus

Protein	Localisation/ interaction	Function
CASP	• *cis*-Golgi apparatus	Tethering protein, communicating with Golgin84
Giantin (372 kDa)	• COPI vesicle; Golgi apparatus membrane • p115, GM130	Golgi apparatus structure maintenance Giantin by formation of inter-cisternal cross-bridges within the complex
GM130 (130 kDa)	• *cis*-Golgi apparatus; ER to *cis*-/ *medial*-Golgi apparatus transport • Rab1, p115, Giantin, GRASP65	*cis*-Golgi apparatus matrix protein for stacking of Golgi apparatus cisternae and a docking receptor in vesicular transport. GM130 specifically interacts with p115, providing a membrane-docking site. Both GM130 and p115 are involved in vesicle tethering to Golgi apparatus membranes. The amino-terminus of GM130 binds to p115, whereas the carboxyl-terminus binds to Golgi apparatus membranes. GM130 competes with Giantin for binding to p115 for p115-Giantin and p115-GM130 interactions mediate independent membrane tethering events. Transport from the ER to the *cis*-/ *medial*-Golgi apparatus compartments requires the action of p115, GM130 and Giantin via a sequential mechanism.
Golgin160 (160 kDa)	• Peripheral Golgi apparatus; distribution similar to p115	Maintenance and reorganisation of the Golgi apparatus structure after mitosis. Involvement in nuclear transport and Golgi apparatus to plasma membrane sorting (Insulin).
Golgin84 (84 kDa)	• *cis*-/ *intra*- Golgi apparatus; retrograde transport • Rab1, p115, GM130	Reorganization of the Golgi apparatus after fragmentation during mitosis and maintenance of Golgi apparatus structure. The N-terminal coiled-coil domain and a single C-terminal transmembrane domain is employed in tethering and docking COPI vesicles to the Golgi apparatus in vesicular transport.
GosR1 (28 kDa)	• *cis*-Golgi apparatus • Stx5, Bet1, membrin, Sec22, Sly1	Integral membrane protein on the Golgi apparatus surface. The N- terminal is exposed to the cytosol and anchored to the *cis*-Golgi apparatus via a carboxyl-terminal hydrophobic tail. GosR1 also is a t-SNARE is involved in ER to Golgi apparatus transport and *intra*-Golgi apparatus transport.

Golgi apparatus

Protein	Localisation/ interaction	Function
p115 (115kDa)	• Secretory pathway • GM130, Giantin	General factor acting within the secretory and endocytic pathways to bind transport vesicles by tethering and docking prior to vesicle fusion. Phosphorylated p115 is localised to the cytosol whereas the unphosphorylated form is associated with membranes mostly of the Golgi apparatus complex bound by its amino terminal region. Upon phosphorylation of p115 (Ser942) it is released from the membranes. Both Giantin and GM130 compete for binding to the C-terminal acidic domain of p115. p115- Giantin and p115-GM130 interactions mediate independent membrane tethering events. p115 on transcytotic vesicles is required for vesicle fusion with the target membrane and vesicular tubular clusters involved in ER to Golgi apparatus transport. Rab1 recruits p115 to coat protein complex II (COPII)-vesicles during budding from the ER where it interacts with a select set of SNAREs.

Coat-proteins

Protein	Localisation/ interaction	Function
Clathrin (91 kDa)	• Cell membrane; coated pit; *trans*- Golgi apparatus	Major protein component of coated vesicles and coated pits. It is involved in intracellular trafficking of receptors and endocytosis of a variety of macromolecules. Adaptins are components of the adapter complexes linking clathrin to receptors in coated vesicles. Clathrin-associated protein complexes are hold to interact with the cytoplasmic tails of membrane proteins leading to selection and concentration. Binding of AP180 to clathrin triskelia induces vesicle assembly into 60-70 nm coats.

SNAREs

Protein	Localisation/ interaction	Function
Bet1 (18 kDa)	• Peri-Golgi apparatus • SNARE-complex: Stx5, Ykt6, GOSR2 and Bet1	Golgi apparatus-associated type IV membrane protein. As a t-SNARE Bet1 forms SNARE-complexes with Stx5, Ykt6 and GOSR2 and is required for vesicular transport from the ER to the *cis*-Golgi apparatus and subsequent membrane fusion between ER-derived vesicles and vesicular tubular clusters (VTCs) or for homotypic fusion of ER derived vesicles. Bet1 is predominantly associated with vesicular spotty structures that concentrate in the peri-Golgi apparatus region but are also present throughout the cytoplasm.

SNARES

Protein	Localisation/ interaction	Function
Mss4	• Ubiquitous	Small GTP-binding protein playing a general role in vesicular transport involved in its regulation. As a GEF partner it interacts with Sce4/Ypt1/Rab1+8, 10 stimulating GDP release of Ypt1 and Rab3A.
NSF (70 kDa)	• Membrane-associated in the prevacuolar endosomal compartment	SNARE involved in vesicular transport from the ER to the Golgi apparatus. Its ATPase activity after interaction with SNAP in complex with Stx1A catalyzes the fusion of transport vesicles within the Golgi apparatus cisternae. Its is employed as a fusion protein for cargo proteins to all compartments of the Golgi apparatus stack independent of vesicle origin also being necessary for dissociation of the Stx5-GOS28 complex
Sec22B (22 kDa)	• ERGIC; Golgi apparatus membrane • SNARE complex: Stx5, GOSR2 and BET1 • SNARE complex: Sec22B or Stx18, Use1L and Sec20L	Vesicle-trafficking protein involved in ER to Golgi apparatus membrane trafficking. It is important for targeting and fusion of ER-derived transport vesicles with the Golgi apparatus complex as well as Golgi apparatus-derived retrograde transport vesicles with the ER. Ykt6 can probably replace Sec22B
VAMP4 (25 kDa)	• Golgi apparatus; TGN • SNARE complex: Stx13, Stx6 and Vti1a	Located to the *trans*-Golgi apparatus network employed in endosomal transport
Ykt6	• Cycling between Golgi apparatus and endosomes • SNARE complex: GOSR1, GOSR2 and Stx5 • SNARE complex: Bet1L, GOSR1 and Stx5	Membrane associated isoprenylated protein with v-SNARE receptor activity. It is employed in the ER to Golgi apparatus transport and also found in the early/ recycling endosome to TGN transport.

ER proteins

Protein	Localisation/ interaction	Function
Calreticulin (55kDa)	Endoplasmic and sarcoplasmic reticulum	Multifunctional protein that acting as a major Ca (2+)-binding (storage) protein in the ER lumen promoting folding, oligomeric assembly and quality control in the ER via the calreticulin/ calnexin cycle (calnexin being localised to the membrane and calregulin to the lumen). Calnexin and calregulin are important for the maturation of glycoproteins in the ER and appear to bind many of the same proteins. Calregulin interacts with newly synthesised glycoproteins in the ER assisting protein assembly and may interact transiently with almost all of the monoglucosylated glycoproteins that are synthesised in the ER. It is also found in the nucleus suggesting a role in transcription regulation (regulation of gene transcription by nuclear hormone receptors).

GTP-ases (small guanine nucleotide-binding proteins)

Protein	Localisation/ interactions	Function
Arf1 (21 kDa)	• Golgi apparatus	Member of the human ARF gene family distantly related to the Ras small molecular weight GTPase family. The family members encode small guanine nucleotide-binding proteins that stimulate the ADP-ribosyltransferase activity of cholera toxin and play a role in vesicular trafficking as activators of phospholipase D. The ARF proteins are categorised as class I (ARF1, ARF2 and ARF3), class II (ARF4 and ARF5) and class III (ARF6) members of each class sharing a common gene organization. Arf1 mediates vesicular transport of molecules from the ER to the Golgi apparatus by regulating vesicle budding. It also plays a key role in maintaining the structural integrity and the overall morphology of the Golgi apparatus. Arf1 is recruited onto membranes bound for transport from a cytoplasmic pool and may facilitate the assembly of protein complexes onto coated vesicles further on promoting protein trafficking among different compartments. It also plays a central role in *intra*-Golgi apparatus transport-modulating vesicle budding and un-coating within the Golgi apparatus complex. Its deactivation induces the redistribution of the entire Golgi apparatus complex to the ER. In its GTP-bound form it triggers the association of coat proteins with the Golgi apparatus membrane. Hydrolysis of Arf1-bound GTP mediated by ARF-GAPs is required for dissociation of coat proteins from Golgi apparatus membranes and vesicles.

GTP-ases (small guanine nucleotide-binding proteins)

Protein	Localisation/ interactions	Function
Rab11A (87kDa)	• Peripheral membrane protein (cell membrane); endosome • Rip11 and StxBP6	Employed in modulation of endosomal trafficking.
Rab1A (23kDa)	• Golgi apparatus • Interaction with YipF5	Binds GTP and GDP and possesses intrinsic GTPase activity unfolding this activity in the transport of newly synthesised proteins from the ER to the Golgi apparatus complex and to secretory vesicles. GM130, p155 and Golgin84 were identified as effectors of the active conformation of Rab1. Golgin84 binds to active Rab1 but not to *cis*-Golgi apparatus matrix proteins. It also recruits p115 located on COPII vesicles.
Sar1	• Early secretory pathway	COPII-associated small GTPase essential for the formation of transport vesicles from the ER. COPI- and COPII-coated vesicles mediate vesicular traffic within the early secretory pathway. The COPII vesicle coat protein in a complex with Sar1 promotes the formation of ER derived transport vesicles that carry secretory proteins to the Golgi apparatus complex.

http://www.ihop-net.org/UniPub/iHOP/
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene
http://ca.expasy.org/

3. 2. 3 "Focussed mini-screen"

After successfully establishing the *L. pneumophila* infection of epithelial cells and proving sufficient protein knock-down in these cells, these methods were combined to construct the following mini screen: Three days prior to infection the cells were transfected at ~50 % confluency to allow further spreading after the agent was added. The *L. pneumophila* Philadelphia01 wt strains were inoculated two days prior to infection. On the day of infection the bacteria were adjusted to MOI of 10 and spread onto the cells. Infection was synchronised by centrifugation. After 90 min incubation extracellular *L. pneumophila* were washed away and killed by the Gentamicin containing fresh medium added. 3 h after infection the cells were lysed using saponin. The free *L. pneumophila* were then plated on BCYE-agar-plates in three different dilutions and incubated. The same lysing procedure was applied after 24 h to the second well plate. 3-4 d later CFU count and comparison of *L. pneumophila* replication was possible.

The Gentamicin-protection-assay employed in this work is a very sensitive method and allows distinction of effects that are very mild. *L. pneumophila* replicate in epithelial cells only up to 1-2 log phases (9-15 *L. pneumophilae*) within 24 h. In 2006, Dorer *et al.* published a siRNA screen on trafficking proteins and their impact on *L. pneumophila* growth in *Drosophila* cells. They could show a down-regulation of *L. pneumophila* replication after knock-down of Arf1 to about 60 %. This information given in *Drosophila* cells we looked for a diminished replication in our cell model after Arf1 knock-down and found even a down-regulation of ~ 70 % (Fig. 3.7). This was then used as a positive control for all following experiments. The graph shown below also compares different control reagents that were transfected: GFP, luciferase and mock (cells only treated with RNAIfect).

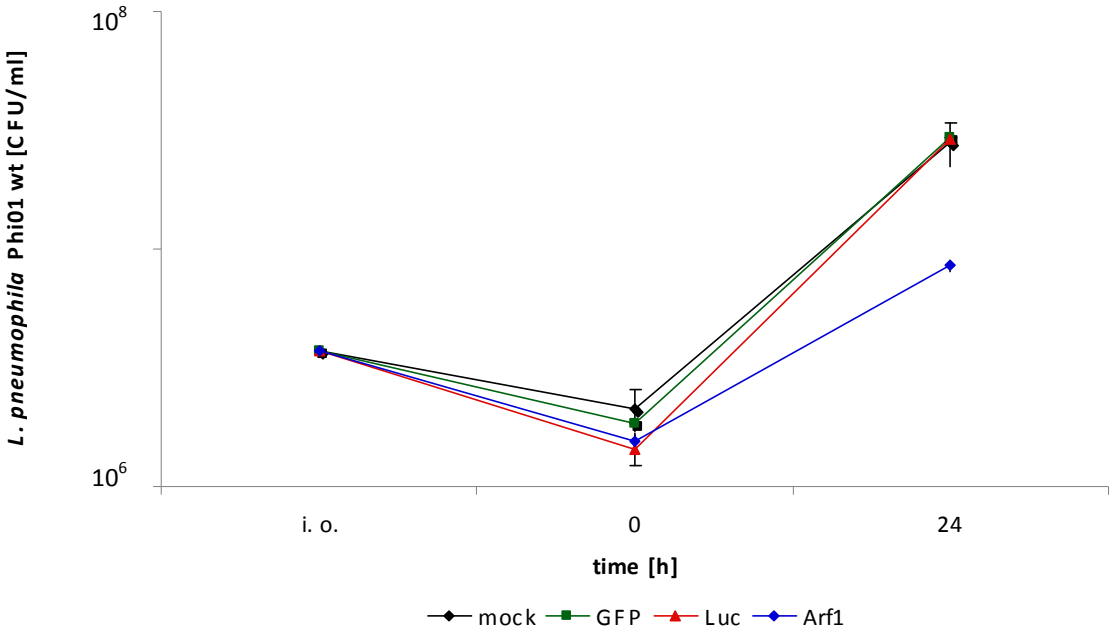

Fig. 3. 7. Replication of *L. pneumophila* Phil01 wt in HeLa model cells transfected with siRNA against GFP, luciferase and Arf1.
Cells were transfected with the depicted siRNAs and 3 days later infected at an MOI of 100. At the given time points of 0 h and 24 h bacterial replication was estimated via CFU count. i. o. is the inoculum. Error bars = standard deviation of triplicates. Results are representative for three independent experiments.

Transfected cells reacted slightly different in *L. pneumophila* uptake but with no significant deviations. Those cells transfected with only the reagent and not containing siRNA gave similar replication-results as those transfected with either siRNA against GFP or against luciferase both having no actual target in eukaryotic cells. In the following experiments these were used as negative controls. In contrast: knock-down of Arf1 resulted in a slightly decreased *L. pneumophila* replication of about 0, 5 log phases (1x 10^6 to 5,9x 10^5). This ~ 60 % replication deficiency is consistent with literature (Dorer *et al.* 2006).

3. 2. 4 *L. pneumophila* replication (model graphs)

In this chapter model graphs for the mini-screen sets are depicted. Each of the sets has been repeated at least three and up to seven times. Each set is only represented by one graph comprising triplets of each knock-down. The results of all experiments were calculated into one graph for *L. pneumophila* infectivity and differed from one experiment to another; replication though was comparable. The experimental set up for the results shown is described under 3. 2. 3 and has been applied to all of the graphs in this chapter.

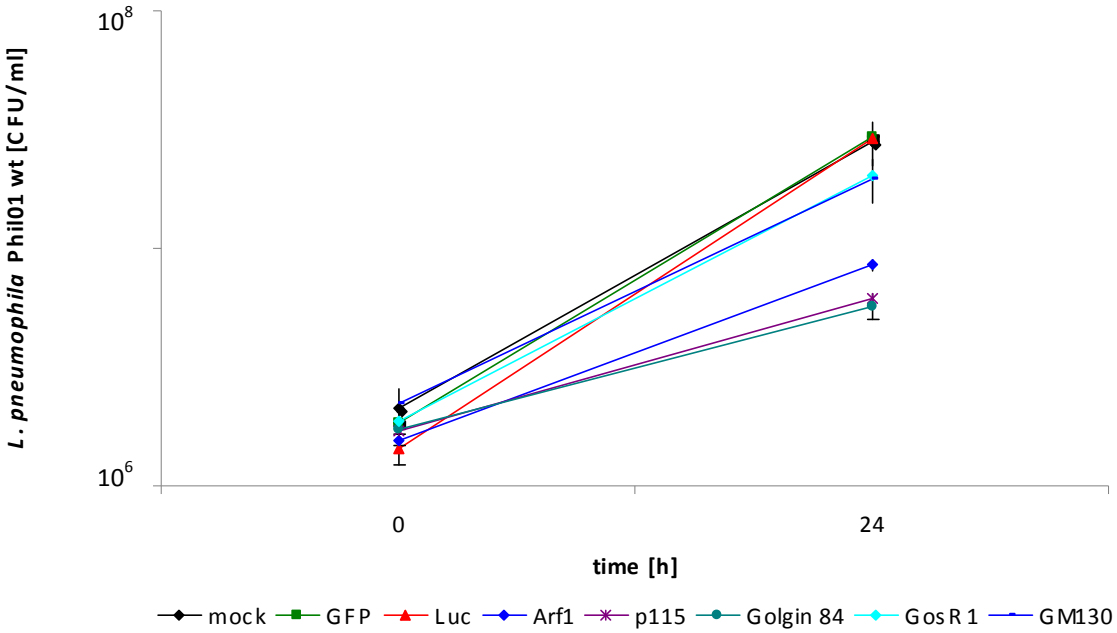

Fig. 3. 8. Different replication of *L. pneumophila* Philadelphia01 wt in HeLa model cells with knock-down of Arf1, p115, Golgin84, GosR1 and GM130, negative controls were GFP, luciferase and mock transfections.
Cells were transfected with the depicted siRNAs and 3 days later infected at an MOI of 10. At the given time points of 0 h and 24 h bacterial replication was estimated via CFU count. Error bars = standard deviation of triplicates. Results are representative for three independent experiments.

This model graph (Fig. 3. 8) shows almost undeterred replication in mock, GFP and luciferase transfected cells. Knock-down of the *cis*-Golgi apparatus-matrix proteins GosR1 and GM130, both employed in vesicle tethering, results in diminished replication efficiency of *L. pneumophila*. Down-regulation of the Golgi apparatus protein Golgin84 and the vesicle tethering factor p115 seem to be even more important for *L. pneumophila* growth within the cell. Their absence resulted in even less replication than in Arf1 deficient cells. Cells treated with Arf1 siRNA showed the down-regulation described prior giving a reliable positive control.

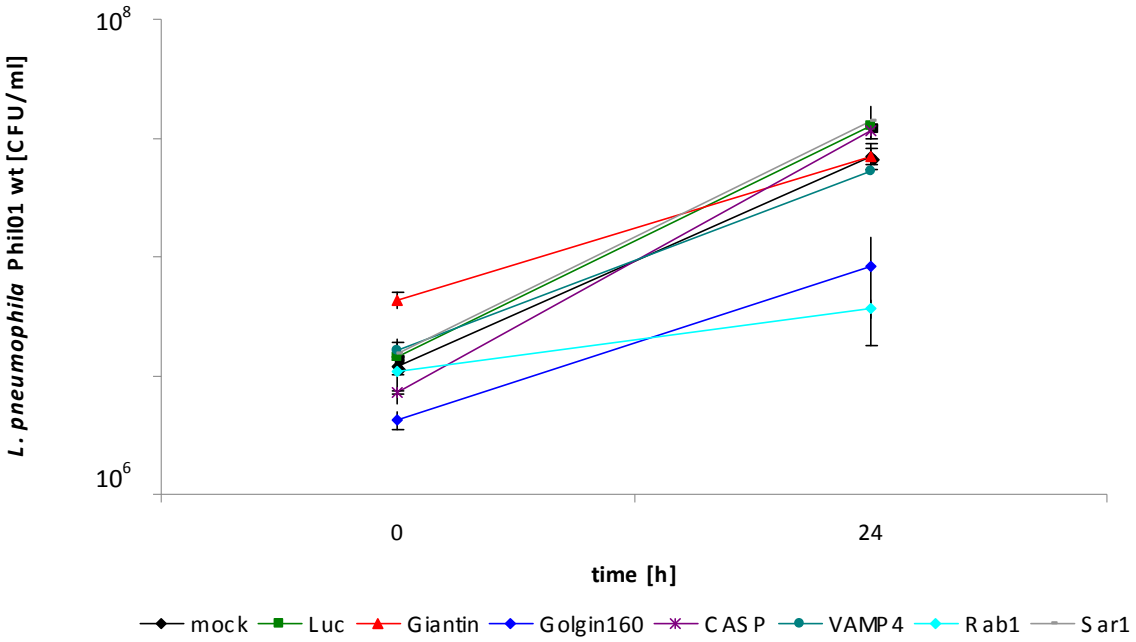

Fig. 3. 9. Replication of *L. pneumophila* Philadelphia01 wt in HeLa model cells with knock down of Giantin, Golgin160, CASP, VAMP4, Rab1 and Sar1, negative controls were luciferase and mock transfections.
Cells were transfected with the depicted siRNAs and 3 days later infected at an MOI of 10. At the given time points of 0 h and 24 h bacterial replication was estimated via CFU count. Error bars = standard deviation of triplicates. Results are representative for three independent experiments.

The graph (Fig. 3. 9) shows varying infectivity rates but mock as well as luciferase gave similar patterns as before, certifying as negative controls. The proteins regulated in this experiment were all related to the Golgi apparatus, especially its structure and also vesicle tethering. Reduction in the Golgi apparatus structure protein Giantin enhanced *L. pneumophila* uptake but impaired its replication by 20 %. Whereby deficiency of Golgin160 inhibited *L. pneumophila* uptake and did also reduce replication by only 20 %. Knock-down of the tether protein CASP (communicating with Golgin84) enhanced replication from 1, 5 to 2 log phases. The SNARE-protein VAMP4 located to the *trans*-Golgi apparatus-network employed in endosomal transport and Sar1 employed in vesicle formation from the ER directed to and from the Golgi apparatus, showed no specific pattern. Rab1 decreased replication by 0, 5 log phases, which is already known from literature (Dorer *et al.* 2006).

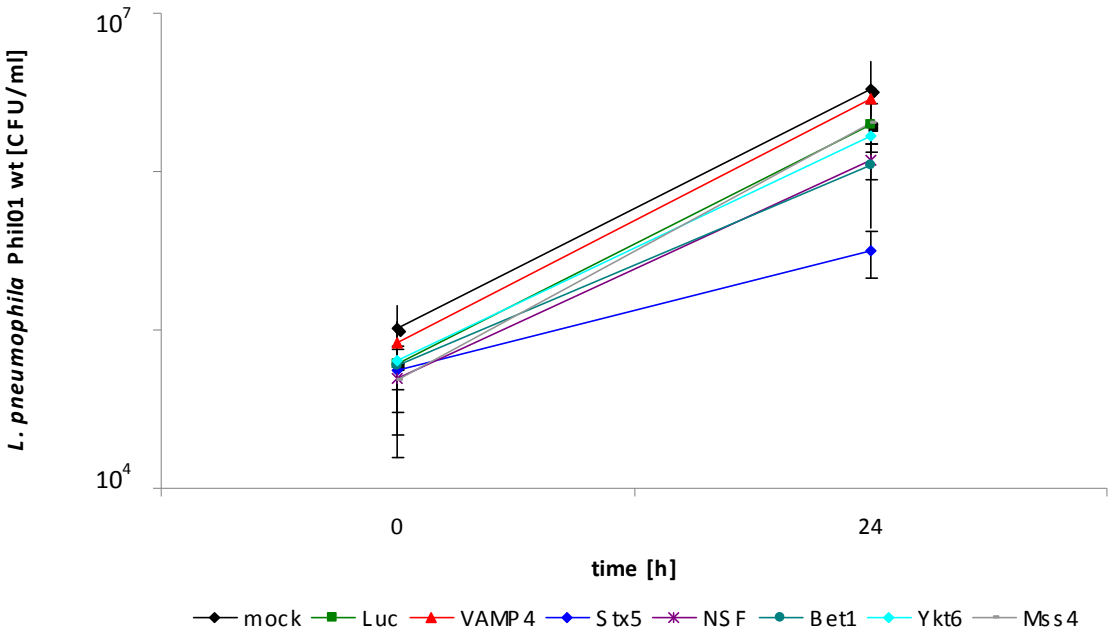

Fig. 3. 10. Replication of *L. pneumophila* Philadelphia01 wt in HeLa model cells with knock down of VAMP4, Stx5, NSF, Bet1, Ykt6 and Mss4, negative controls were luciferase and mock transfections.
Cells were transfected with the depicted siRNAs and 3 days later infected at an MOI of 10. At the given time points of 0 h and 24 h bacterial replication was estimated via CFU count. Error bars = standard deviation of triplicates. Results are representative for three independent experiments.

All other proteins included in this graph (Fig. 3. 10) are so called SNARES responsible for correct vesicle transport, tethering and fusion. Protein knock-down of Bet1, a type IV membrane protein required for vesicular transport from ER to Golgi apparatus, could later not be verified (Meissner unpublished) so this curve gives no actual reaction to Bet1 deficiency. Ykt6 has SNARE receptor activity in the endosomal pathway but its absence in this case does not significantly influence *L. pneumophila* replication. Mss4 a small GTP-binding protein involved in regulation of intracellular vesicular transport here also does not seem to be essential for *L. pneumophila* replication. The only SNARE-protein that actually had an impact on replication (within this setting) – when absent – was the t-SNARE Stx5 functioning in ER to Golgi apparatus transport and in *intra*-Golgi apparatus homotypic vesicle fusion. Its absence resulted in reduction of intracellular growth of more than 0, 5 log phase. Taken together the SNARE proteins seem to be very redundant in their functions and one might take over the function of another so that *L. pneumophila* replication is not impaired. The only SNARE protein important enough, to show a reaction is Stx5 which is not only employed in the ER to Golgi apparatus transport, but also in the transport and fusion of *intra*-Golgi apparatus homotypic fusion, important for Golgi apparatus homoeostasis.

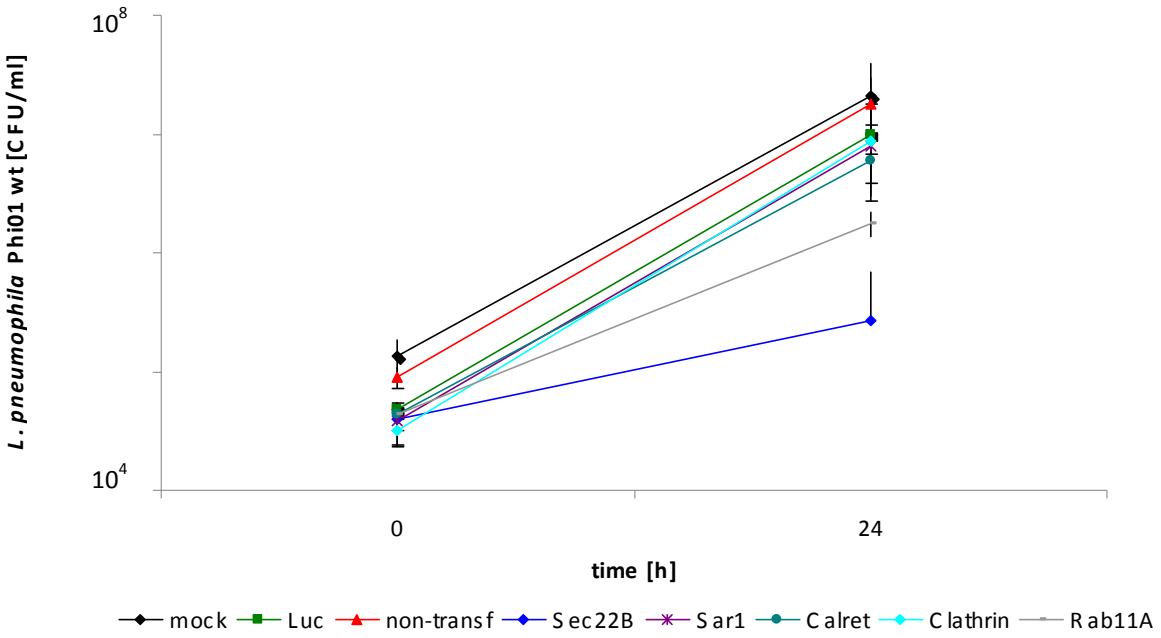

Fig. 3. 11. Different replication of *L. pneumophila* Philadelphia01 wt in HeLa model cells with knock-down of Sec22B, Sar1, calreticulin, clathrin and Rab11A, negative controls were mock, luciferase and non-transfected cells.
Cells were transfected with the depicted siRNAs and 3 days later infected at an MOI of 10. At the given time points of 0 h and 24 h bacterial replication was estimated via CFU count. Error bars = standard deviation of triplicates. Results are representative for three independent experiments.

This graph (Fig. 3. 11) represents a set of ER connected proteins employed in structure, vesicle formation and transport. Both, mock- and luciferase-transfected cells were used as negative controls, plus infection of un-transfected cells. These were not even treated with the mock reagent. Here too *L. pneumophila* replication was as efficient as in the other negative controls. The ER resident SNARE Sec22B, a vesicle-trafficking protein in ER to Golgi-apparatus transport, located to the ERGIC (ER-Golgi apparatus intermediate compartment) and to the Golgi apparatus membrane, is known to be very important for *L. pneumophila* growth (Derré & Isberg 2004). We could even show a down-regulation of *L. pneumophila* replication in the absence of Sec22B of up to 90 %. Derré & Isberg 2004 and Kagan *et al.* 2004 showed that calnexin, Sec22b, and Rab1 are recruited to the *L. pneumophila* containing vacuole LCV. Bet1 was tested here too, but the knock-down was not sufficient (Meissner unpublished). The absence of Sar1, an ER associated SNARE employed in COPII vesicle formation and anterograde transport from the ER directed to the Golgi apparatus, here did not show any interference with *L. pneumophila* replication. This was not expected, as the theory implicates a specific interception of the anterograde pathway by *L. pneumophila*. Here again SNARE redundancy might be an explanation. In this case Sar1 was not specifically tested for protein knock-down, this might be another explanation. Calreticulin plays a role in assisting protein assembly and in retaining unassembled protein subunits in the ER; its absence did not disturb *L. pneumophila* growth inside the cell. Down regulation of clathrin, a major protein component of coated vesicles and coated pits,

travelling from the cell membrane to the *trans*-Golgi apparatus back and forth, did not interfere with *L. pneumophila* growth in this setting. But absence of Rab11A seemed to have a drastic influence on *L. pneumophila* replication reducing it about 80 %. Rab11A is one of the proteins of the large Rab GTPase family having regulatory roles in the formation, targeting and fusion of intracellular transport vesicles and being located to the peripheral cell membrane and endosomes.

3. 2. 5 Fold of replication

All data from the graphs combined and calculated into fold of replication resulted in the graph depicted below. Every single experiment was calculated into fold of replication of the grown bacteria in comparison to the bacterial burden in luciferase transfected cells. Replication in luciferase-transfected cells was taken to be 100 %; all other ranges were calculated accordingly.

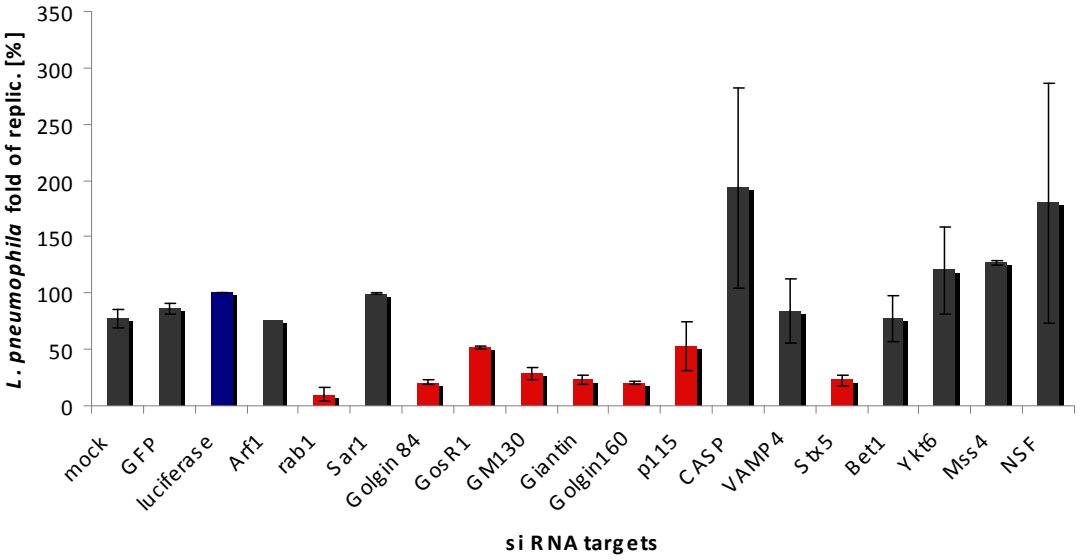

Fig. 3. 12. Fold of *L. pneumophila* Phil01 wt replication in HeLa model cells treated with the siRNAs depicted. This graph combines all experiments including the models shown above of *L. pneumophila* replication in transfected HeLa model cells, derived from the described Gentamicin assay. On the x-axis are depicted the proteins down-regulated by siRNA: Arf1, Rab1A, Sar1, Golgin84, GosR1, GM130, Giantin, Golgin160, p115, CASP, VAMP4, Stx5, Bet1, Ykt6, Mss4 and NSF. The y-axis represents % of *L. pneumophila* replication, calculate dependant on luciferase given as 100 %, after protein-down-regulation. Error bars = standard deviation of at least three up to seven independent experiments. Red bars stand for replication down-regulation of more than 50 %. Green bars mark up regulated *L. pneumophila* replication.

The over-view of the efficiency of *L. pneumophila* replication after down-regulation of specific trafficking proteins including the positive controls Arf1 and Rab1 shows a distinct pattern (Fig 3. 12). As the Gentamicin-protection-assay is very sensible and highly assured by its quantitative outcome even mild effects of a very redundant system like intracellular trafficking after pathogen infection might well be studied. As could be shown, the importance of the Golgi-apparatus upon *L. pneumophila* infection is really underestimated. It is very well described that ER derived vesicles are

hijacked by the LCV, but our findings also suggest that Golgi-apparatus structure and vesicular trafficking to and inside the Golgi-apparatus is important for this pathogen. The graph shows a 50 % - 80 % down-regulation of bacterial burden in cells lacking the structural Golgi compartment proteins: Golgin84 (20, 9 % replication), GM130 (29 % replication) and Giantin (22, 6 % replication). Also the integral- / peripheral-Golgi apparatus proteins GosR1 (50, 9 % replication) and Golgin160 (20 % replication) were necessary for replication. The absence of tethers like p115 (52, 8 % replication) and SNAREs like Stx5 (22, 8 % replication) that interact with Rab1 (10 % replication) were also important for *L. pneumophila* replication. Arf1, as the best described interaction partner was robust in 76 % replication down-regulation.

3. 2. 6 Putative interaction-site of *L. pneumophila* effector LidA with the SNARE-complex

After the finding of Stx5, the SNARE protein in the anterograde pathway (ER to Golgi-apparatus) and in the *intra*-Golgi transport, being so important for *L. pneumophila* replication that its down regulation resulted in only 22,8 % bacterial growth, compared to the luciferase control, we decided to further investigate this *in silico*. Stx5 builds a SNARE complex with Bet1, membrin and Sec22B. Sec22B investigated in this work also gave a result connecting it to *L. pneumophila* replication (10 % replication). Bet1 was tested too for its impact on bacterial replication, but as the siRNA later turned out not to efficiently down-regulate protein amount (Meissner unpublished), this non-impact on replication might not give a realistic answer.

All SNARES so far are known to have a specific coiled-coil domain, the so called SNARE motif. These motifs are characterised by a heptad repeat pattern in which residues in the first and fourth position are hydrophobic and residues in the fifth and seventh position are predominantly charged or polar. This pattern can be used to predict SNARE motifs in amino acid sequences. One *L. pneumophila* protein associated with maintenance of bacterial integrity and with putative function in trafficking is LidA. It has been shown that two translocated substrates, SidM (also known as DrrA) and LidA, target host cell Rab1. SidM is a guanosine nucleotide exchange factor for the inactive form of Rab1 recruiting it to the LCV. LidA enhances this process, but how exactly it does so is yet unknown. From *Chlamydia* research it is known that the property of the IncA protein (secreted *Chlamydia* effector) to assemble into multimeric structures has been connected to a co-evolution with the SNARE machinery for a role in membrane fusion (Delevoye *et al.* 2004). Here we could show that *in silico,* LidA (amino acid sequence Fig 3. 13) has the same potential to form these stable coiled-coil structures that would enable SNARE interactions. The putative SNARE motif region is stained grey.

LidA:

```
        10         20         30         40         50         60
MAKDNKSHQV KTSEGSLQSV KTKEKEPVVE KMRVEDSKKE DKLSMPTTKK ESQPNEPVKP
         70         80         90        100        110       #49
FKTSFEKWIE SSLLDPQAKE DRGSTINLGR EGLKNASQVK KFLLSPAGKD VIAELGAQMA
        130        140        150        160        170        180
LQRNINLQNQ QDRMEHELFK RRLMAALFLW YLSKKSHAAE KVKEIIREYN EKAIKNAEKA
        190        200        210        220        230        240
SKPSQKSTSS TAQADKEIQK MLDEYEQAIK RAQENIKKGE ELEKKLDKLE RQGKDLEDKY
        250        260        270        280        290        300
KTYEENLEGF EKLLSDSEEL SLSEINEKME AFSKDSEKLT QLMEKHKGDE KTVQSLQREH
        310        320        330        340        350        360
HGIKAKLANL QVLHDAHTGK KSYVNEKGNP VSSLKDAHLA INKDQEVVEH EGQFYLLQKG
        370        380        390        400        410        420
QWDAIKNDPA ALEKAQKDYS QSKHDLATIK MEALIHKLSL EMEKQLETIN DLIMSTDPEK
        430        440        450        460        470        480
NEEATKLLHK HNGLNLKLAN LHDMLAVHRK EKSFFNEKGE EVTSLNDAHY VIGKDQQLFN
        490        500        510        520        530        540
LGGKFYPIHK EQKILEKDGK FYLLKQGEDW ESIKDSPEKQ KKAEHDFHKL QYETPMTVKK
        550        560        570       #36580        590        600
LVHHNKGLET TIHKERIEET KQQLEDNGKE KIEIANNISK LQSTVGTALN ELNQSNINQE
        610        620       #58       #39        640       #61       #42
SVTLTPLGGG GTTLSPKPGP SLAAVTTFFR TKIQEMKEMN SPGMTRDELR QFQSQIPEGA
   #62  670        680        690        700        710        720
ARNYFISALT QMPRTGPVPF QIMQAMLRNL ERFGVDTTKP GVTSIRSKTD EVVEQRFNPT
        729
PSLSPFKTS
```

Fig. 3. 13. Amino acid sequence of LidA.
Sequence stretches marked in grey depict the putative coiled-coil regions used in the alignment below (Swiss-Prot/TrEMBL Q5WXW8).

When over-expressed in mammalian cells or yeast, LidA interferes with the early secretory pathway, probably via a domain predicted to be rich in coiled-coil structure (Derré & Isberg 2005). LidA is secreted early and stays expressed throughout the replication cycle (Derré & Isberg 2005). The prediction of coiled-coils in the sequence of LidA was verified by computational analysis using the http://www.coiled-coil.org site (see Fig. 3. 14).

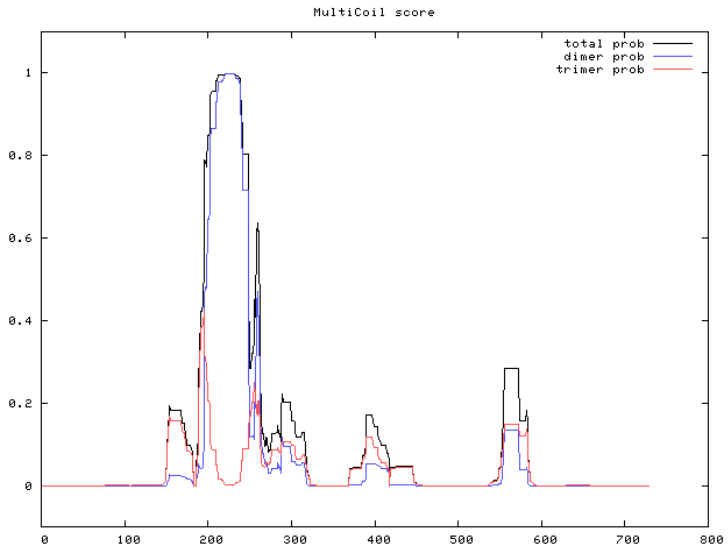

Fig. 3. 14. Graphical output of the computational analysis of multi coiled-coil domains of LidA**.**
L. pneumophila LidA sequence (from Swiss-Prot/ TrEMBL #Q5WXW8) is predicted to form potential dimeric coils between amino acids 190 and 280.

Analysis of the LidA amino acid sequence showed similarity with the coiled-coil domain of the eukaryotic fusion machinery, the SNARE domain. For this work, we aligned

several 53 amino acid stretches of the LidA sequence with the four helices of the SNARE domains of the known SNARE complex of Stx5, membrin, Bet1 and Sec22B (Fig 3. 15). Hydrophobic amino acids are coloured in blue, hydrophilic residues are green. Basic amino acids are stained grey in this model. In this alignment, the helices are connected by layers of mostly hydrophobic amino acids with the exception of the hydrophilic central layer, like in the SNARE complex.

Putative LidA-Stx5 SNARE interaction:

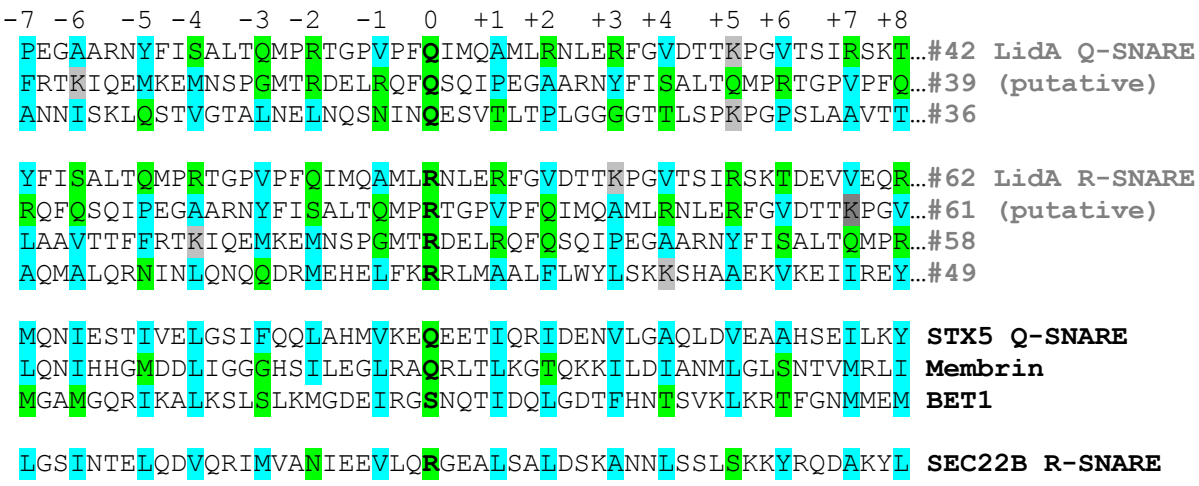

Fig. 3. 15. Sequence alignment of the four-helix bundle region of a putative SNARE complex of *L. pneumophila* LidA and *human* Stx5 motif**.**
Blue and green depict the amino acids forming the four-helix bundle in the synaptic fusion complex. The conserved glutamine (Q) and arginine (R) residues forming the ionic 0 layer are indicated in green. The alignment with the SNARE complex was done manually based on predicted hydrophobic and hydrophilic amino acids. Hydrophobic amino acids (aa) are coloured in blue, basic aa are grey, the central hydrophilic residue is marked in green. (alanine A, arginine R, asparagine N, cysteine C, glutamine Q, glycine G, histidine H, isoleucine I, leucine L, lysine K, methionine M, phenylalanine F, proline P, serine S, threonine T, tyrosine Y, valine V). The LidA-sequence is derived from Swiss-Prot/TrEMBL #Q5WXW8; Stx5, membrin, Bet1 and Sec22B are to be found at Swiss-Prot/TrEMBL #Q13190.

Altogether, the modelling shows that two stretches of the LidA protein sequence are compatible with the formation of very stable four parallel helix bundles, resulting in a structure similar to the SNARE complex. This leads to a model involving SidM as the GEF for Rab1, LidA as one SNARE component competing with Stx5, membrin, Bet1 or Sec22B for fusion and a tether not yet identified for localisation of this complex to a specific membrane. Further experiments are needed here to confirm this theory.

3. 2. 7 Localisation of specific ER- and Golgi apparatus-proteins in *L. pneumophila* infection

The results from the “mini-screen” suggested an involvement of the Golgi apparatus as well as the ER in *L. pneumophila* infection. The importance of the ER in *L. pneumophila* phagosome construction is

well described. In between the ER and the Golgi-apparatus a small organelle is found, the so called ERGIC. Several proteins are accumulated and sorted into vesicles that bud at ER exit sites and fuse to form the tubular vesicular ER–Golgi apparatus intermediate compartment (ERGIC). Clusters of the ERGIC then move along microtubules. The next station is the Golgi apparatus where they fuse to the *cis*-Golgi apparatus. ERGIC-53 is a specific marker protein for this compartment.

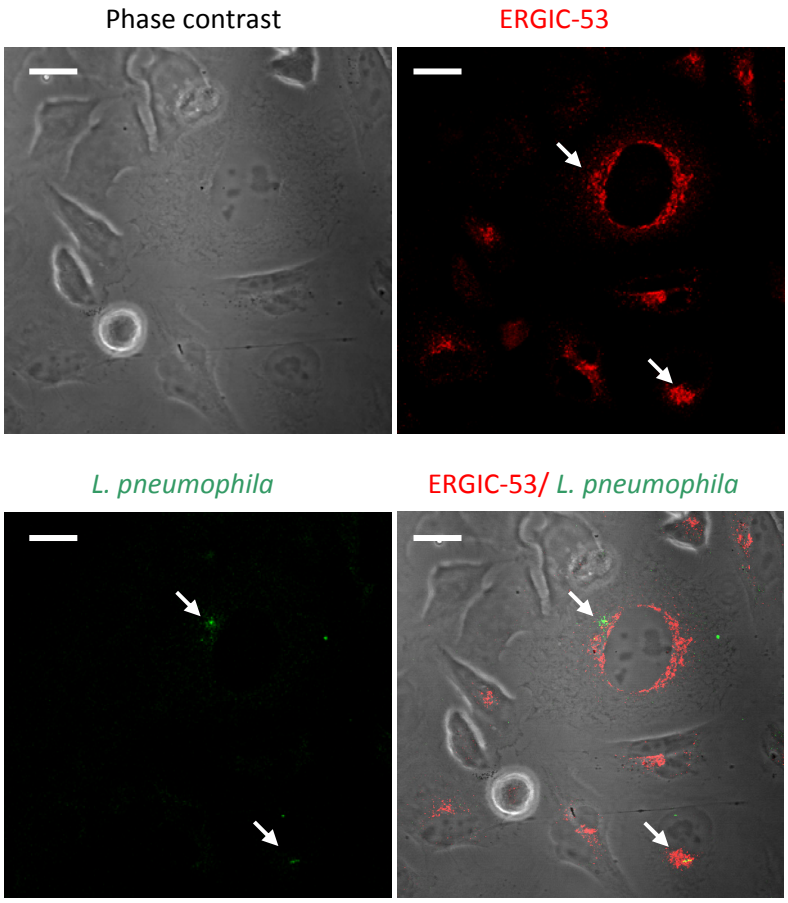

Fig. 3. 16. ERGIC-53 immunostaining of *L. pneumophila* Philadelphia01 wt infected HeLa model cells 20 h post infection.
Upper left is a phase contrast picture; upper right shows the red ERGIC-53 fluorescent staining. In the lower left picture, the *L. pneumophila* are stained green. The lower right picture shows the merge. Two different patterns of ERGIC staining can be distinguished: In the bigger cell above (arrow), the ER surrounding the nucleus is stained (*L. pneumophila* stained here are on top of the cell). In the lower right corner (arrow) the ERGIC staining surrounds the *L. pneumophila* and almost all ERGIC-53 is accumulated around the phagosome. Scale bars 10µm.

The immunofluorescence-staining of ERGIC-53 depicted in figure 3. 16 shows two different patterns the proteins distribution in an infected cell. As there was no Gentamicin in the cell medium, the time point of infection is not defined. The cells were incubated with the pathogen for up to 20 h. In the big cell above, the protein is distributed in the complete ER surrounding the nucleus. Here two *L. pneumophila* can be detected stained green attached to the cell. All of the other cells show normal ERGIC staining in only the compartment itself. In the cell on the lower right the *L. pneumophila* containing phagosome is completely surrounded by ERGIC-53. This finding is consistent with the

theory of the LCV hijacking ER derived vesicles to stud its phagosome. Additionally it shows that the traffic interception localises not only to the ER, but also to the ER intermediate compartment and might even disturb the succeeding compartment, the Golgi apparatus.

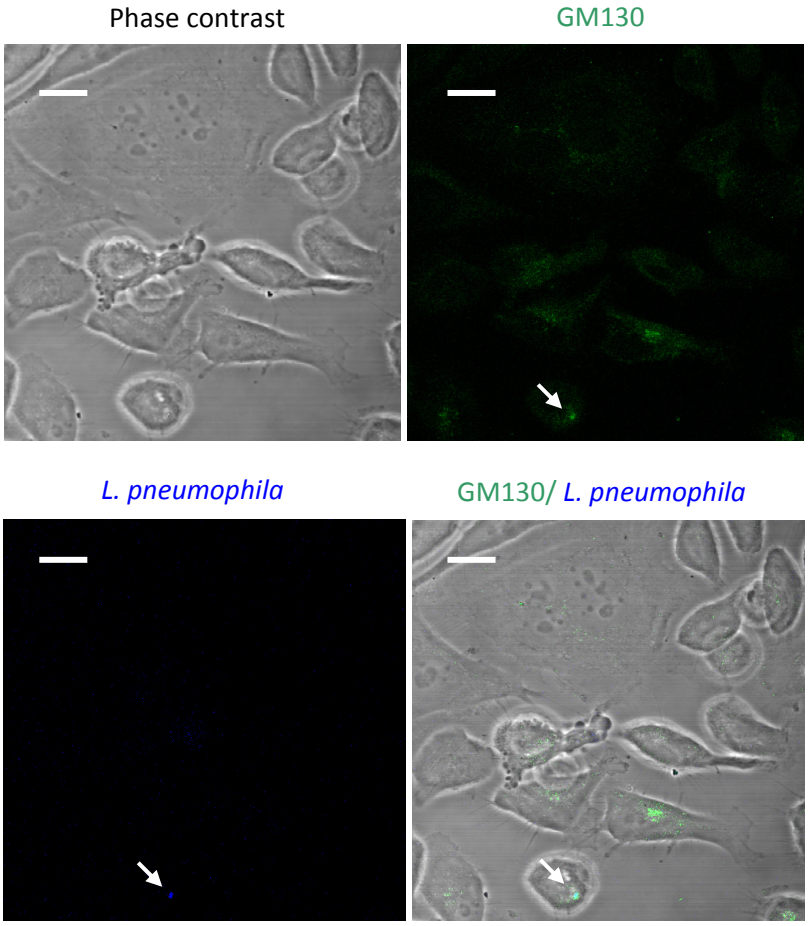

Fig. 3. 17. GM130 immunostaining of *L. pneumophila* Philadelphia01 wt infected HeLa model cells at 20 h post infection.
Upper left is a phase contrast picture; upper right shows the green GM130 fluorescent staining. In the lower left picture, the *L. pneumophila* are stained blue. The lower right picture shows the merge of the three. GM130 is a Golgi apparatus matrix protein, specifically staining the Golgi apparatus (seen in the upper cell). In the cell in the lower centre (arrow) GM130 co localises with the *L. pneumophila* stained blue. Scale bars 10µm.

GM130 is a *cis*-Golgi apparatus matrix protein providing a membrane-docking site, specifically in p115 mediated tethering and docking of transport vesicles to their target membrane. In the microscopy picture above, GM130 is stained green, localizing to the Golgi apparatus. The *L. pneumophila*, stained blue, in the lower centre of the picture, clearly co-localises with the GM130 Golgi apparatus staining (Fig 3. 17). This states at least a possible interaction of the pathogen with the Golgi apparatus.

3. 2. 8 Summary

All results from the *L. pneumophila* replication assay upon specific protein knock-down of host-cell trafficking proteins; the *in silico* modelling of a *L. pneumophila* effector into a putative SNARE-complex with host-cell proteins and the visible co-localisation of ER and Golgi compartment proteins with the *L. pneumophila* phagosome underline the multiple ways of cross talk of the pathogen with its host on the secretory pathway. After particularly investigating the role of Golgi apparatus proteins, there being matrix proteins, vesicle SNAREs and tethers localised all over the Golgi apparatus it can be stated that the impact of this organelle on *L. pneumophila* infection has been underestimated. Many of the structure proteins of the Golgi apparatus and proteins trafficking at the *cis*-Golgi apparatus seem to be important for *L. pneumophila* growth, whereas TGN proteins do not have such a big impact on the pathogens replication. Most of the Golgi apparatus proteins investigated have coiled-coil structures that either anchor them to the membrane and/ or enable them to interact with other proteins, mostly in the manner of SNAREs or tethers. Knock-down of the proteins Golgin84, Golgin160 and Giantin are known to result in fragmentation of the Golgi structure (Heuer *et al.* 2009). Down regulation of these proteins showed a comparably severe impact on *L. pneumophila* replication, as well as Rab1, GM130 and Stx5, which leads to the suggestion, that the dispersed Golgi apparatus somehow hinders the pathogen in constructing of its replication niche. The co-localisation of GM130 with the phagosome does also seem to somehow connect the phagosome to the Golgi apparatus. The impact of Rab1 and Stx5 on the pathogens replication is severe and almost comparable. The mechanism in which *L. pneumophila* hijacks Rab1 is well described until now, but the way, how exactly it employs Stx5 has to be further investigated. It can be stated that the phagosome must well recognise the SNARE residue of Stx5, maybe by use of effectors and so employing it for its own needs.

Here we could show that even the ER-Golgi apparatus intermediate compartment (ERGIC) is attached to the phagosome. But we could not verify in our studies that Sar1 knock-down has any impact on *L. pneumophila* replication though it is an important protein in ER to Golgi apparatus transport. Sar1 similar to Arf1 assists in COP vesicle formation at the ER directed to the Golgi apparatus. The knock-down of Sar1 in this work was verified only on mRNA level, but the actual amount of protein was not tested. So the observed effect might be due to the fact, that the protein level was not impaired.

3. 3 Protein-phosphorylation detection and characterisation

Protein phosphorylation, the most prevalent reversible covalent modification, is a regulatory mechanism in virtually every metabolic process in eukaryotic cells. Infection of eukaryotic cells with bacteria alters host cell functions, and protein phosphorylation is known to be a part of the associated molecular changes. Intracellular bacteria have evolved survival strategies for manipulating host cell signalling pathways to establish beneficial replicative niches within their host. Here protein phosphorylation is only one example of a broad range of tools by which effectors target key host proteins to remodel signalling events targeting a wide range of effects.

Two specific ways are known by which *L. pneumophila* effectors manipulate their host on basis of phosphorylation activities. One is its manipulation of Rab-GTPase function as for example RalF exchanging Arf1´s phosphate (Nagai *et al.* 2002). The other is the modulation of phospho-inositide. SidC for example binds to PI (4) P on the vacuole (Luo & Isberg 2007). Other bacteria interact with their host via phosphorylation and subsequent reorganisation of the whole cytoskeleton here only to name two *Listeria* and *Shigella*.

Since protein phosphorylation plays a key role in cellular signalling it was an interesting question to see whether *L. pneumophila* infection caused phosphorylation events on host cell proteins. Phosphorylation in eukaryotic cells is grouped in phosphoryl additions to threonine, serine and tyrosine, so one of the three known possibilities could specifically be investigated. The following project is based on observations by Coxon *et al*. 1998 and Susa *et al*. 1999. They showed induction of tyrosine protein phosphorylation in MRC-5 cells after *L. pneumophila* invasion 24 hours post infection, as well as activation of tyrosine kinase and protein kinase C and induction of actin polymerisation in human monocytes.

In this approach, we wanted to obtain a first overview over tyrosine-phosphorylations in *L. pneumophila* infection. That is why we used *L. pneumophila´s* natural host, the protozoa, as model of infection. *Acanthamoeba castellanii* should definitely show phosphorylation pattern after infection with wt *L. pneumophila*.

3. 3. 1 Phospho-Tyrosine detection in *L. pneumophila* infected *Acanthamoeba castellanii*

To investigate the impact of cellular *L. pneumophila* infection, on tyrosine phosphorylation, we chose an approach employing the pathogens natural host. In this part of the work we infected 2x 10^8 cells *Acanthamoeba castellanii* with different *L. pneumophila* wt strains: Corby wt and Philadelphia wt at an MOI of 10. The amoebae were grown in 6-well-plates in specific medium. Before infection the cells were starved off glucose for a few minutes and *L. pneumophila* wts Corby or Philadelphia were added onto the ~60 % confluent amoeba mono-layer diluted in the starvation medium. This

approach did not imply centrifugation or antibiotics. After two different time points 1, 5 h and 5 h post infection the cell layer was harvested in loading dye. The samples were denaturised at 98˚C and the proteins separated on a SDS-PAGE. Subsequent immunoblotting and detection of proteins with a specific phospho-tyrosine antibody gave the following Western Blot picture (Fig. 3. 18).

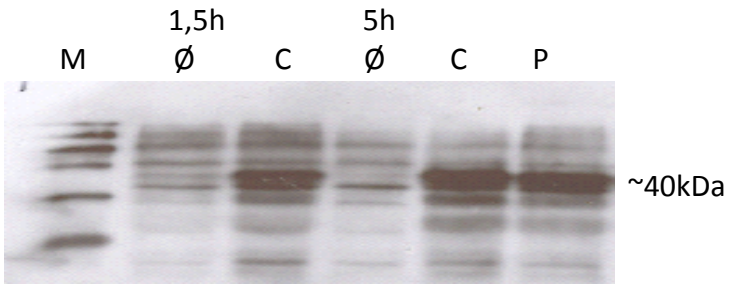

Fig. 3. 18. Tyrosine phosphorylated proteins in *A. castellanii* infected with *L. pneumophila* Corby wt (C) and Philadelphia01 wt (P) and uninfected (Ø).
Amoebae were infected with *L. pneumophila* at an MOI of 10. After 1, 5 h and 5 h the amoeba were harvested on ice and denaturised in phosphorylation inhibiting loading dye. After electrophoretic separation and blotting, proteins were detected with a phospho-tyrosine specific antibody (P-Tyr).

The phospho-tyrosine specific antibody detects a protein band at ~40 kDa that is very much enhanced in cells infected with any of the *L. pneumophila* wild types used. This is the case at 1, 5 h after infection compared to uninfected cells. The band is still visible 5 hours after infection with Corby wt as well as with Philadelphia01 wt. From this blot we cannot say whether the protein is enhanced or only phosphorylated but we succeeded in purifying the protein for further analysis.
A comprehensive analysis of protein phosphorylation within one cell (phospho-proteomics) involves identification of phospho-proteins and phospho-peptides, localization of the exact residues that are phosphorylated and quantification of phosphorylation.

3. 3. 2 Purification and identification of the detected phospho-tyrosine containing protein

Purification and identification of proteins with tyrosine phosphorylation sites in *L. pneumophila*-infected *Acanthamoeba castelanii* was monitored using the PhosphoScan Kit and subsequent LC MS/ MS identification of the peptide. *A. castellanii* was infected with *L. pneumophila* Corby wt at an MOI of 10 and subsequently incubated for 6 hours. The samples were then purified. The assay is based on the specific enrichment of phospho-tyrosine - containing peptides using an antibody against phospho-tyrosine. Cells were lysed in a urea-containing buffer, and cellular proteins were digested by protease and fractionated by reversed-phase solid-phase extraction. Peptides were then subjected to immuno-affinity purification using phospho-tyrosine mouse antibody coupled to protein G-agarose beads. Over night incubation ensured high-affinity binding of phospho-tyrosine-containing peptides

to the beads. Unbound peptides were removed through washing, and phospho-tyrosine containing peptides were eluted with dilute acid. Reversed-phase chromatography was performed on micro tips to separate phospho-peptides from antibody and to concentrate them for LC tandem mass spectrometry. The LC-tandem mass spectrum (LC/ MS/ MS) was done at the proteomics-core facility at the institute. Phospho-peptides are generally difficult to analyze by mass spectrometry (MS) for several reasons. They are negatively charged whereas electro spray is generally performed in the positive mode. Being hydrophilic, they do not bind well to columns that are routinely used for purification of peptides before analysis. Phospho-peptides are not observed as intense peaks, especially in the presence of other non-phosphorylated peptides owing to ionic suppression. Finally, if the protease produces peptide fragments that are too small or too large, the peptides might not be observed in the mass spectrum at all (Mann *et al.* 2002). The tandem MS analysis run, using three samples obtained by the procedure described above resulted in the following spectrum (Fig. 3. 19).

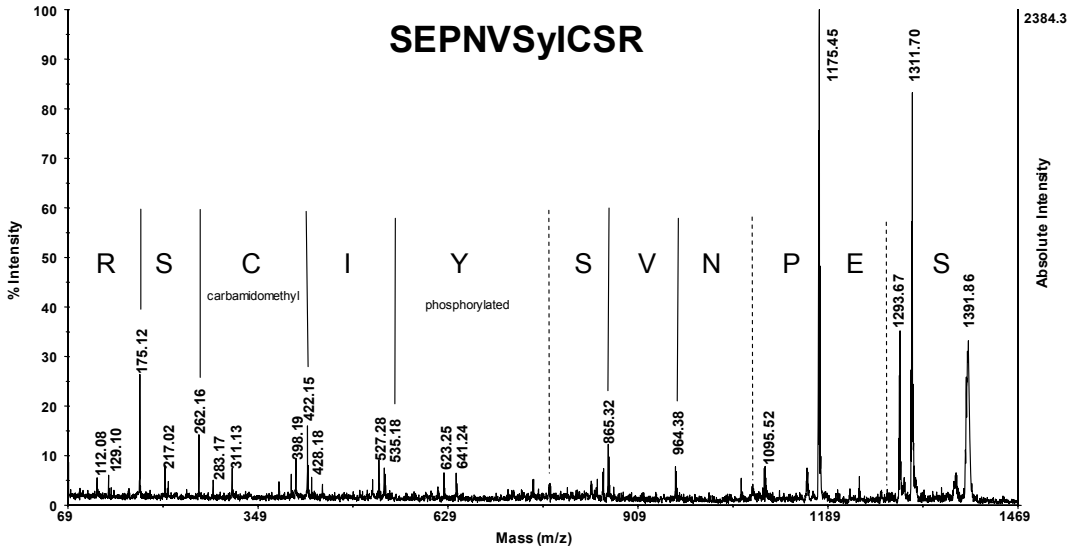

Fig. 3. 19. The LC MS/ MS spectrum of a peptide isolated using the P-Tyr kit.
The spectrum depicts a peptide of 1391, 54 Da mass. The assigned peaks are marked with straight lines. Dotted lines stand for peaks that are not shown in this spectrum. This picture is merged from three different experiments. The phosphorylated tyrosine is marked by use of small letter.

The three samples run on LC MS/ MS resulted in a congruent peptide sequence: SEPNVSYICSR. The peptide contains the phosphorylated tyrosine (Y) that had been looked for. Blasted on the *Entamoeba* genome – the only amoeba genome available today - the search resulted with a score of 39, 2 in a protein sequence of 386 amino acids. This is annotated to be a serine/ threonine protein kinase of a calculated molecular weight of 44 kDa with a catalytic domain, sequence shown in figure 3. 20a:

***Entamobea* serine-threonine-kinase:**

```
MSKRLLKRVS QTEVFQNYGI QEGIILDNET NKYRISKVIG YGAFGIVFLA TNIKTGEVNA
IKRVSQGKRF KNKELRIQEI LHHQNVIKLK DSFLSKLSNS SSFCLNLVMD YLPQNLYQFM
RSLKKKVPTI YIRLFSYELI RGLAYLHSLN IIHQDIKPEN ILVNKNTGDL KICDFGSAKN
ILDSSEPNVS YICSRHYRAP ELVFRTSKYN SSIDIWSYGC ILAEMFLGKP LFPGSSTSDQ
VVKIIKIIGT PTEEEMKAMN NEIPPPILPK VDGIGIENTL SSFKPPVQAI TILTHTLQYS
PEKRLSASKL LTSEFHKELF NEGVLLPNGN PIPILTQYDE DEWKRGKENE TIDKMKEFVE
GEKKRILSQN SIRKDKETKD IISDAK
```

Fig. 3. 20a. Protein sequence resulting from the SEPNVSYICSR peptide-BLAST on the *Entamoeba* genome. The 386 amino acid sequence is annotated to a serine-threonine-kinase containing the tyrosine residue. The peptide is marked in red. Score 788 bits, E-value 0.0. Source: NCBI protein BLAST.

The serine-threonine-protein kinase of the serine or threonine-specific kinase subfamily with a catalytic domain is annotated to be a phospho-transferase. The enzymatic activity of these protein kinases is controlled by phosphorylation of specific residues in the activation segment of the catalytic-domain - the sought after tyrosine-residue. Phosphorylation is sometimes combined with reversible conformational changes in the C-terminal auto regulatory tail. Activation then enables further phosphorylation as a cascade protein. An alignment of the detected *Enteamoeba* protein sequence with the *human* genome resulted in a protein sequence with homology to glycogen synthase kinase 3 (GSK3) (Fig. 3. 20b).

***Entamobea* serine-threonine-kinase + *Human* glycogen-synthase-kinase-3:**

```
YRISKVIGYGAFGIVFLANNIKTGEINAIKRVSQGKRSKNKELRIQEMLHHQNVIKLKDS  entamoeba
Y  +KVIG G+FG+V+ A    +GE+ AIK+V QGK  KN+EL+I   L H N+++L+
YTDTKVIGNGSFGVVYQAKLCDSGELVAIKKVLQGKAFKNRELQIMRKLDHCNIVRLRYF  human

FLSKLSNSSSFCLNIVMDYLPQNLYQFMRSL---KKKVPIIYIRLFSYELIRGLAYIHSL  entamoeba
F S         LN+V+DY+P+ +Y+  R     K+ +P+IY++L+ Y+L R LAYIHS
FYSSGEKKDEVYLNLVLDYVPETVYRVARHYSRAKQTLPVIYVKLYMYQLFRSLAYIHSF  human

NIIHQDIKPENILVNKNTGDLKICDFGSAKNILDSSEPNVSYICSRHYRAPELVFRTNKY  entamoeba
 I H+DIKP+N+L++ +T  LK+CDFGSAK ++   EPNVSYICSR+YRAPEL+F    Y
GICHRDIKPQNLLLDPDTAVLKLCDFGSAKQLV-RGEPNVSYICSRYYRAPELIFGATDY  human

NSSIDIWSYGCILAEMFLGKPLFPGSSTSDQVIKIIKIIGTPTEEEMKAMNNEIPPPILP  entamoeba
 SSID+WS GC+LAE+ LG+P+FPG S  DQ+++IIK++GTPT E+++ MN        P
TSSIDVWSAGCVLAELLLGQPIFPGDSGVDQLVEIIKVLGTPTREQIREMNPNYTEFAFP  human

KVDGIGIENTLRSFKPPSQAIQILTHTLQYSPEKRLSASRLVTSEFHKELFSEGVLLPNG  entamoeba
++         R   PP +AI + +  L+Y+P  RL+        F  EL    V LPNG
QIKAHPWTKVFRPRTPP-EAIALCSRLLEYTPTARLTPLEACAHSFFDELRDPNVKLPNG  human

NPIPLLTQYDENEWKRGKENETI  entamoeba
   P L  +   E        TI
RDTPALFNFTTQELSSNPPLATI  human
```

Fig. 3.20b. Sequence alignment of the E*ntamoeba* serine-threonine-kinase to *human* GSK3. *Entamoeba* serine-threonine-synthase aligned to its *human* homolog chain A of glycogen synthase kinase 3 results in a score of 288 bits and identities of 43 % (positives = 201/323 (62%), gaps = 5/323 (1%)). Source NCBI protein BLAST 2 sequences.

This detected GSK3 also contains the putatively phosphorylated tyrosine, but with a slightly different peptide sequence, starting with G instead of S. GSK3 is a proline-directed serine-threonine-kinase that was initially identified as a phosphorylating and inactivating glycogen synthase. GSK3 is involved in energy metabolism, neuronal cell development, and body pattern formation (Plyte *et al.* 1992). This kinase phosphorylates and inactivates the glycogen-synthase connected to the wnt-signalling cascade through β-catenin. It is itself regulated by phosphorylation via the protein-kinase-B. The wnt-signalling cascade describes a network of proteins well studied for their roles in cancer for example, but also involved in normal physiological processes in adult animals (Lie D. C. *et al.* 2005).

3. 3. 3 Summary

The search for a specific protein phosphorylated after *L. pneumophila* infection resulted in the finding of a 44 kDa protein kinase. The phospho-tyrosine protein we found in *Acanthamoeba* was not a *L. pneumophila* effector protein, but most likely a kinase involved in the general signalling pathways of the cell. This hints to a large impact of the pathogen in all different cellular information pathways and should be investigated further.

3. 4 NF-κB activation and detection after *L. pneumophila* infection

As stated before, the intracellular pathogen *L. pneumophila* interacts with its host cell in many different ways to promote its own survival. The pathogens ability to replicate within the cell depends on its own survival inside the cell as well as the cells survival until bacterial replication is completed. Above, we enlisted specific trafficking proteins that are employed to support *L. pneumophila´s* phagosome formation this being administered by effectors specifically interacting with the host cell machinery (SidM and Rab1). Other bacterial proteins also translocated by the type IV secretion system are also shown to be crucial. For example two distinct effectors are known to particularly be involved in inhibiting macrophage cell death: SdhA and SidF (Laguna *et al.* 2006, Banga *et al.* 2007). It is known, that *L. pneumophila* specifically interacts with the anti-apoptotic pathway controlled by the mammalian nuclear transcription factor-κB (NF-κB). NF-κB stands for a family of transcription factors that link extracellular stimuli to cellular responses thereby controlling inflammation, innate immunity, cell division and survival (Karin & Greten 2005, Hoffmann & Baltimore 2006, Hayden & Ghosh 2008). It is evident that one or more of the type IV-dependant effectors must be involved in activation of the NF-κB regulated pathway.

NF-κB is an interesting protein to study anti-apoptosis. For this purpose an assay has been constructed that detects NF-κB activation via green fluorescent protein (GFP). The model shall be summarised shortly. A construct of GFP labelled p65 was used for screening NF-κB activation in

epithelial cells. p65 is usually resident in the cellular cytoplasm. The anti-apoptotic cascade of NF-κB is not activated as long as it is bound to the inhibitor IKκB. Upon degradation of this inhibitor p65 is transduced into the nucleus where it binds to the DNA and initiates transcription of anti-apoptotic molecules (Fig. 3. 21).

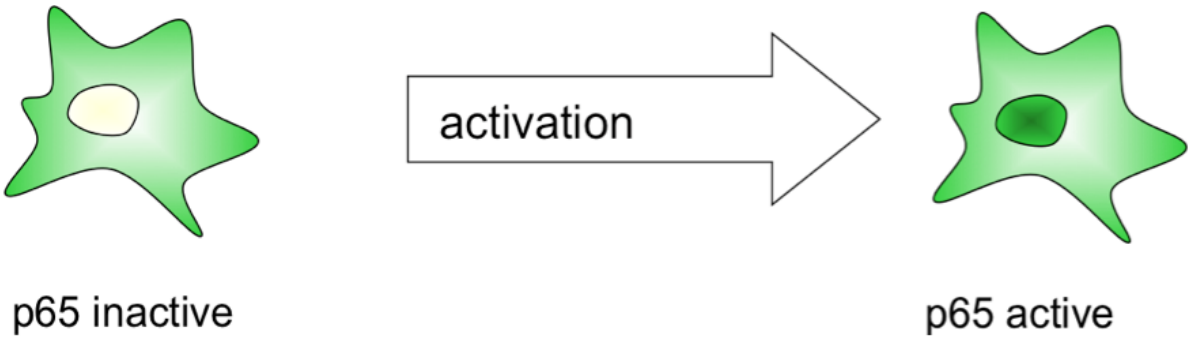

Fig. 3. 21. Schematic figure of the NF-κB activation assay.
Inactive GFP marked p65 localizes to the cytoplasm of the cell. Upon activation it translocates to the nucleus to enhance transcription. The nucleus is then stained green. Read out by automated microscope defines nucleus and cytoplasm and measures green fluorescent intensity, therewith concluding activation or non-activation (adapted from Bartfeld *et al.* 2009).

The assay detects the fluorescent p65 and defines its location in- or outside the nucleus. A read out of fluorescent cells via the automated microscope can distinguish between GFP staining of the cytosol and the signal inside the nucleus. The green fluorescent nucleus was termed “activated” in contrast to an inactive DAPI-blue stained nucleus.

The “NF-κB-activation-assay” was then combined with *L. pneumophila*-infection-assay described above to monitor anti-apoptotic response to infection. The focus lay on *L. pneumophila* mutants lacking specific effectors and their impact. The mutants *ΔsdbA* and *ΔsdhA* for example are defective in single *L. pneumophila* effectors of the type IV secretion system.

Biphasic NF-κB activation after *L. pneumophila* infection

The nuclear translocation of green fluorescent p65 can be read out by the automated microscope and quantified. A time course of infection was run, leading to the observation of a distinct NF-κB activation pattern over a time period of up to 8 hours; two peaks characterised this. The first peak at about 70 minutes after *L. pneumophila* infection decreased after 120 minutes. This was followed by a constant activation over the remaining 7 hours, shown in the schematic figure 3. 22a.

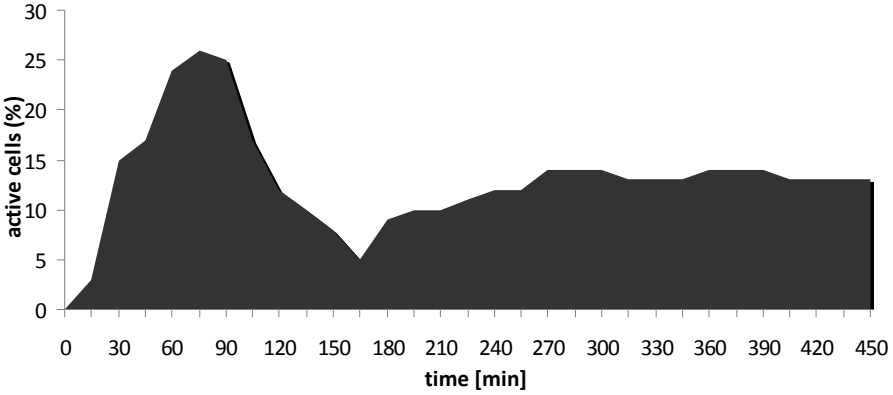

Fig. 3. 22a. NF-κB activation upon *L. pneumophila* infection.
A549 cells with p65GFP (A549 SIB01) were infected with Philadelphia01 wt and the GFP translocation measured over time, depicting two phases of p65 nuclear translocation. The experiments were done in cooperation with Sina Bartfeld, who constructed the NF-κB activation assay.

On the one hand side it is known that NF-κB activation is dependant on TLR5 and its adaptor MyD88 (Hawn *et al.* 2003, Losick *et al.* 2006). On the other hand it is common knowledge that the structural component of the bacterial flagellum, flagellin, induces IL-8 secretion via NF-κB in epithelial cells (Schmeck *et al.* 2006, Teruya *et al.* 2007). Knowing that flagellin of *L. pneumophila* is recognised by TLR5, the next evident step was, to test the mutant strain *ΔflaA*, defective in the pathogens flagellum. This mutant did only activate NF-κB in the long term. The *ΔdotA* mutant defective in the type-IV secretion system, in contrast did only shortly induce activation, in the pattern of the first peak, as shown in figure 3. 22b.

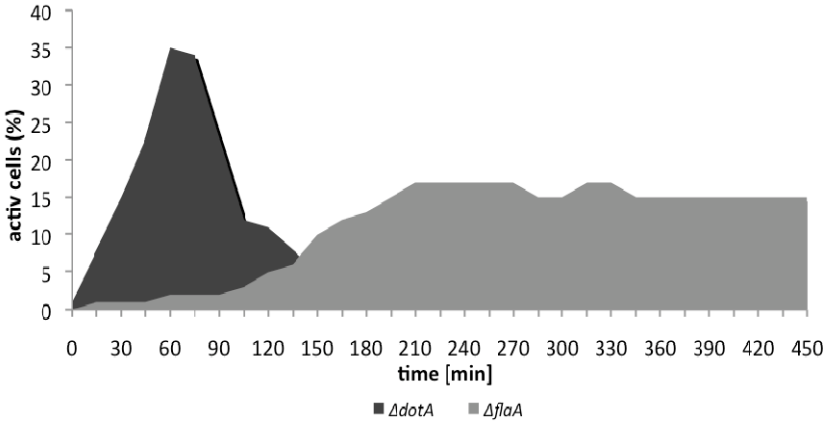

Fig. 3. 22b. NF-κB activation upon infection with *L. pneumophila* mutants.
A549 cells with p65GFP (A549 SIB01) were infected with Corby and JR32 mutants *ΔflaA* and *ΔdotA* and the GFP translocation measured over time, depicting two phases of p65 nuclear translocation. The experiments were done in cooperation with Sina Bartfeld, who constructed the NF-κB activation assay

From this it was deduced, that the first peak was due to the cell's recognition of the pathogen's flagellum. This is usually triggered by TLR-5 sensing of flagellin and further activation of MyD88 and

the subsequent pathway. The long-term activation is dependant on the type IV secretion system. Mutants lacking the Dot/ Icm translocation system were not able to activate NF-κB in the long term and were not able to replicate inside their host cell. This was connected with their incapability to activate the anti-apoptotic pathway.

3. 4. 1 Growth of *L. pneumophila* Corby wt and Phil01 wt in HeLa and A549 model cells

As the *L. pneumophila*-infection-assay described above (in chapter 3.2) was set up in HeLa (human cervix adeno-carcinoma) model cells and the NFκ-B assay was constructed of A549 (human lung-epithelial cells), it was necessary to find out, whether these cell types showed differences in *L. pneumophila* replication. In figure 3.23 we compared *L. pneumophila* replication in the HeLa and A549 model cell lines. Cells were infected with the *L. pneumophila* wt strain Philadelphia01 at am MOI of 100 for 24 h.

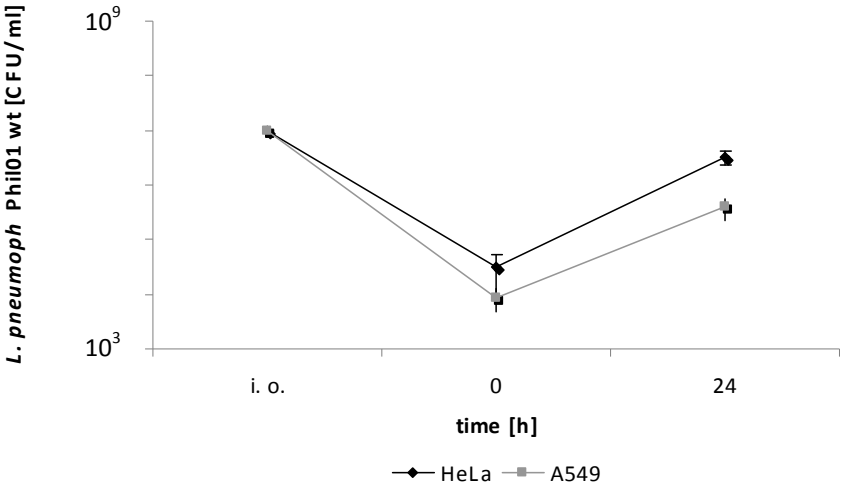

Fig. 3. 23. Replication of *L. pneumophila* Philadelphia01 wt in HeLa and in A549 model cells.
Cells were infected at an MOI of 100. Amount of replicated bacteria was estimated by CFU count. Error bars = standard deviation of triplicates. Results are representative for three independent experiments.

The Philadelphia01 wt showed the same efficiency of replication in both of the tested cells at an MOI of 100 within 24 h. Though infectivity was not comparable, A549 as well as HeLa model cells allowed replication over 1, 5 log phases. The HeLa model cells were even more susceptible to infection than A549 cells.

3. 4. 2 *L. pneumophila* mutants *ΔsdbA* and *ΔsdhA* growth in comparison

From the NF-κB activation assay described above and subsequent analyses employing *L. pneumophila* mutants, two interesting mutants defective in Dot/ Icm effectors came up, and that gave an unusual activation pattern. One of these was SdhA (NF-κB activation pattern in Fig. 3. 24a).

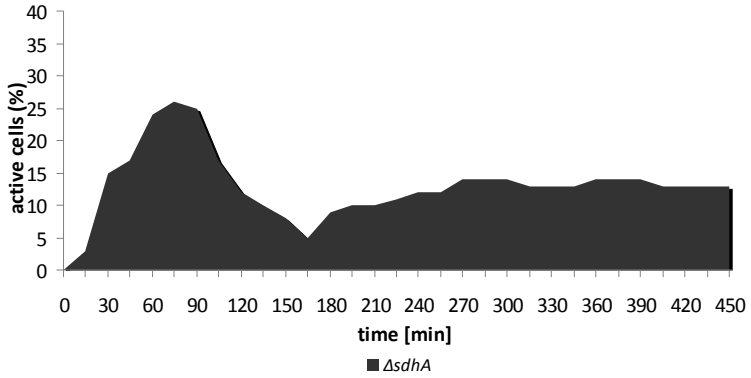

Fig. 3. 24a. Schematic figure on NF-κB activation pattern of *L. pneumophila* Philadelphia *ΔsdhA* mutant in SIB01 A549 cells over time. These experiments were done by Sina Bartfeld.

This effector is known to be deficient in activation of anti-apoptosis (Laguna *et al.* 2006). The other mutant was deficient in the effector SdbA (Fig. 3 25a). This protein and its function are not yet well characterised. What is known is that it contains 3354 bp (giving 1117 aa) and that it is a putative substrate of the Dot/ Icm system with a calculated molecular mass of 127075 Da (sequence SdbA from: http://genolist.pasteur.fr/LegioList). To further investigate these two effector-mutants a growth assay in epithelial cells was designed. Here growth of *ΔsdhA* and *ΔsdbA* mutants was compared to wt *L. pneumophila* and those lacking the Dot/ Icm system (shown as a control).

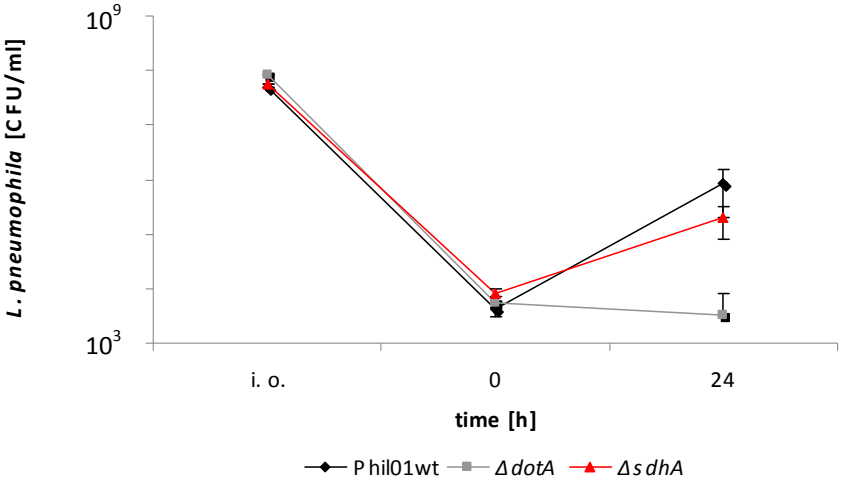

Fig. 3. 24b. Replication of *L. pneumophila* Philadelphia01 wt, Philadelphia *ΔdotA* mutant and Philadelphia *ΔsdhA* mutant in A549 cells.
Cells were infected at an MOI of 100. Amount of replicated bacteria was estimated by CFU count. Error bars = standard deviation of triplicates. Results are representative for three independent experiments.

In A549 cells the Philadelphia *ΔsdhA* mutant shows diminished replication in comparison to the Philadelphia wt (Fig 3. 24b). Replication of the Philadelphia *ΔdotA* mutant even decreased, as usual. This is explained by the fact that *L. pneumophila* exiting the cell are killed in the Gentamicin-containing medium. These findings underline the results of this mutant's replication being reduced in mouse bone marrow derived macrophages. This experiment shows that this is also true for cells from the human system.

The effector SdbA is not as well described as SdhA. The mutant lacking SdbA did show even less replication in the given epithelial cell model. The bacterial burden of Philadelphia *ΔsdbA* mutant stayed consistent in comparison to a decrease in the Philadelphia *ΔdotA* mutant's burden, but did not show any replication at all (Fig 3. 25b).

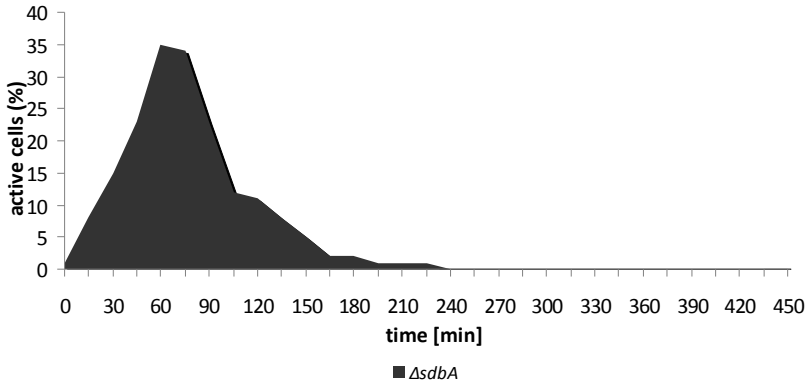

Fig. 3. 25a. Schema of NF-κB activation pattern of *L. pneumophila* Philadelphia *ΔsdbA* mutant in A549 SIB01 cells over time. These experiments were conducted by Sina Bartfeld.

Since the mutant lacking the complete Dot/ Icm system again was killed after exiting the cell, *ΔsdbA* must either have at least had some replication or some anti-apoptotic signalling, because the bacterial burden did not grow less.

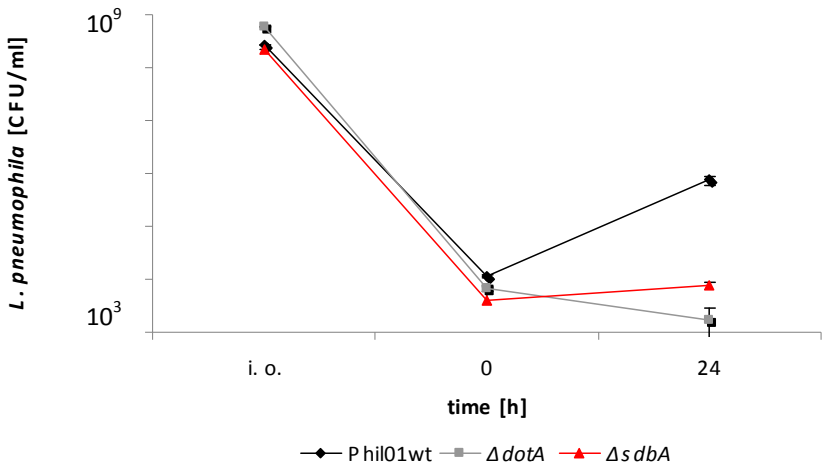

Fig. 3. 25b. Growth of *L. pneumophila* Philadelphia01 wt, Philadelphia *ΔdotA* mutant and Philadelphia *ΔsdbA* mutant in A549 cells.
Cells were infected at an MOI of 100. Amount of replicated bacteria was estimated by CFU count. Error bars = standard deviation of triplicates. Results are representative for three independent experiments.

3. 4. 3 Summary

Replication of *L*. *pneumophila* defective in SdbA and SdhA in A549 cells is consistent with their activation or lack of activation of NF-κB. *L. pneumophila* lacking SdhA grew better than the other mutants though not as well as the wt stain. In the NF-κB activation-assay this effector mutant could still activate NF–κB. Where as *L. pneumophila* defective in SdbA did not replicate as did DotA deficient ones. This again is mirrored in their complete lack of p65 activation.

4 Discussion

4. 1 *L. pneumophila*-infection model

The bacterium *L. pneumophila* has been termed an "accidental human pathogen" by Chien *et al.* in 2004. It was recognised as a human opportunistic pathogen after its isolation from patients in an outbreak of fatal pneumonia (Legionnaire's disease) at an American Legion convention in Philadelphia (Fraser *et al.* 1977). The intracellular pathogen employs a Dot/ Icm type IV secretion system (Vogel *et al.* 1998, Segal *et al.* 1998 and 1999, Zamboni *et al.* 2003, Segal *et al.* 2005) to deliver a set of effector proteins into its host cytosol (Nagai *et al.* 2002, Luo *et al.* 2004, Chen *et al.* 2004). These effectors then modulate the fate of the phagocytic vacuole containing *L. pneumophila* (LCV) by preventing phagosome-lysosome fusion and vacuole acidification. In addition, it recruits vesicles derived from the endoplasmic reticulum (ER) (Coers *et al.* 1999, Nagai *et al.* 2003). This particular procedure leads to the mechanistic suggestion that the LCV hides behind an ER-wall. Also, while replicating inside the cell, the pathogen induces anti-apoptotic signalling to bar its host from suicide (Laguna *et al.* 2006, Abu-Zant *et al.* 2007, Banga *et al.* 2007). After successful proliferation within the LCV, the bacteria destroy the host cell and proceed to infect other cells.

L. pneumophila only being one of several intracellular pathogens, it seems worth while to take a closer look at its genetics in order to understand its position within the bacterial world. Indeed 60 % of the *L. pneumophila* genes have homologs among phylogenetically diverse intracellular bacteria (*Coxiella*, *Salmonella*, *Chlamydia*, *Rickettsia*, *Brucella*, and *Mycobacterium* species). This is comparable to the 63 % homology found in seven fully sequenced related, γ-proteobacteria. This suggests that the species´ similar life-styles and common origin may be rooted in gene complement similarity (Chien *et al.* 2004). Of the phylogenetically diverse bacteria, the intracellular bacteria *Legionella*, *Brucella*, *Salmonella* and *Chlamydia* are known to target the secretory pathway and establish a specific replication niche. These similarities in genome and intracellular life-style made it an interesting task to compare *L. pneumophila* to the genetically not so closely related *Chlamydia* (*Chlamydiae*), which displays a comparable intracellular life-style, and the closer related *Salmonella*, which also replicates within the cell and interferes with the host's secretory pathway. Heuer *et al.* in 2009 showed that interaction with the Golgi apparatus (GA) structure is crucial for *Chlamydia* intracellular replication. *Salmonella* (also a γ-proteobacterium like *L. pneumophila*) being closer related also build a phagosomal vacuole (*Salmonella* containing vacuole – SCV) in interaction with the Golgi apparatus (Holden 2002). The physiological significance of Golgi apparatus localisation to the SCV is still unclear but disruption of the Golgi apparatus network resulted in strong inhibition of *S. typhimurium* replication in epithelial cells (Salcedo & Holden 2003). In contrast, fragmentation of the Golgi structure enhanced *Chlamydia* replication. Up until now it was only known that *L. pneumophila* hijacks ER derived vesicles to surround its vacuole (Nagai *et al.* 2003), which makes the results of our study interesting insofar as the role of the Golgi apparatus had not yet been analysed.

In this study we constructed a "mini-screen" to sort out the specific proteins hijacked by *L. pneumophila* to establish its vacuole in order to provide space for replication. We established a *L. pneumophila*-infection model in epithelial cells, which we could later manipulate effectively by RNA interference.

The first step in setting up the *L. pneumophila* infection was to find out how the wild type (wt) strains of *L. pneumophila*, Corby, JR32 and Philadelphia01 and the *ΔdotA* mutant in JR32 replicated under varying conditions: amount of bacteria given as multiplicity of infection (MOI) of 10 and 100; period of time (3 h, 24 h, 48 h and 72 h) and cell type (HeLa and A549 cells) for ideal replication. A time course was designed and varying MOIs were compared in two different cell types of epithelial cells. These initial experiments establishing the replication amounts in epithelial cells demonstrated that *L. pneumophila* do not very efficiently infect epithelial cells. From initially applied 5x 10^7 bacteria only 3x 10^4 were detected in the cell 3 h after infection. The wild type strains showed replication of only 1 to 2 log phases. On the other hand, the Dot/ Icm deficient bacteria mutant *ΔdotA* did not show any growth at all. Their detectable amount even decreased. We infer this being due to the bacteria being released into the Gentamicin containing medium outside the cells because of earlier cell deaths. This could be explained by the caspase-3 activation and subsequent apoptosis of the cell because no effectors could be translocated into the cytosol by the Dot/ Icm translocation system, preventing apoptosis (Gao & Abu Kwaik 1999a, b, 2000). We subsequently used this growth deficiency as a negative control for intracellular replication of *L. pneumophila*. The multiplicity of infection needed for successful replication of the *L. pneumophila* wild type strains was as high as 10. Even infection with an MOI of 100 did not result in higher replication. We therefore decided to use MOI of 10 as being most physiological. The screen consisted of two parts: As a first part we selected the *L. pneumophila* wt strain Philadelphia01 to infect the HeLa model cells since throughout the infection procedure they delivered the most constant results. This experimental set-up seemed most suitable for the mini-screen. Second part of the screen was the effective manipulation of the host cell via RNA interference, to down-regulate target proteins.

4. 2 *L. pneumophila* hijacks its host trafficking pathway & the role of Golgi apparatus structure

The identification of host cellular components targeted by different pathogens is an interesting means to provide insight into the molecular mechanisms mediating bacterial interactions with the secretory pathway and may additionally increase our understanding of the normal functioning of the secretory pathway. Intracellular pathogens have developed three distinct strategies to survive phagosome-lysosome fusion. Two of these mechanisms, tolerance of the toxic environment and escape from the phago-lysosome into the cytosol, are used by a variety of pathogens such as *Salmonella enterica* and *Listeria monocytogenes*, respectively. *L. pneumophila, Chlamydia* and several other intracellular pathogens use a third strategy: prevention of phagosome-lysosome fusion. In this work we concentrate on *L. pneumophila* and its survival strategies inside its host, but we kept our eyes open on the strategies of other intracellular pathogens like *Chlamydia* and *Salmonella* as well. The most intriguing aspect of these bacteria's and especially of *L. pneumophila´s* biology is their extraordinary ability to commandeer the organelle trafficking system of a wide range of host cells.

After uptake of *L. pneumophila* by its host cell, the bacteria are found within a specialised vacuole (LCV) that does neither fuse with lysosomes nor acidify (Horwitz *et al.* 1983, 1984), thus allowing for replication. The specialised LCV associates with secretory vesicles (Coers *et al.* 1999), mitochondria, and rough ER (Horwitz *et al.* 1983, Kagan *et al.* 2002) and - near the end of the replicative cycle - acquires late endosomal markers (Sturgill-Koszycki & Swanson 2000). These results strongly suggest that the bacteria play an active and continuous role in modulating organelle trafficking events from within the limits of their specialised vacuole. Not unexpected for an intracellular pathogen, examination of its genome indicates that *L. pneumophila* has undergone horizontal gene transfer (Chien *et al.* 2004). Several homologs of eukaryotic like proteins were identified (Brüggemann *et al.* 2006), including the previously described RalF (Nagai *et al.* 2002) and SidM (DrrA) (Machner & Isberg 2006, Murata *et al.* 2006) both being guanosine triphosphatase (GTPase) modifiers, recruiting host GTPases to the *L. pneumophila* containing vacuole. However, *L. pneumophila* mutants lacking the above mentioned RalF, SidM or LidA effectors do not display obvious defects in organelle trafficking or intracellular replication (Nagai *et al.* 2002, Conover *et al.* 2003).

These observations already pose broad hints at the severity of the problem when attempting to give simple answers to questions on trafficking and intracellular replication. The redundancy of the system made it almost impossible to obtain satisfying results with the methods given (see comments by Ensminger & Isberg 2009). Replication deficiencies upon protein knock-down were minute. Down regulation of Rab1, a protein definitely known to be important for *L. pneumophila* replication, decreased replication only by 0, 5 log phase. Other authors also published these minimal impacts during the time of this work (Dorer *et al.* 2006). Needless to say, that this poses a challenge on the accuracy and reproducibility of the assays used.

The results of this work are based on observations obtained from the *L. pneumophila*-Gentamicin-infection assays as described above. This model is well established in host-pathogen interaction studies and has been repeated in each case for at least three times (each time including triplicates) and up to seven times in some cases. As a positive control we used knock-down of Arf1, as this protein is published to specifically interact with the *L. pneumophila* effector RalF. In 2006, while these experiments were run, Dorer *et al.* also confirmed this finding; defining 40 % less replication upon Arf1 knock-down. In our experiments Arf1 knock-down reproducibly resulted in a robust 76 % so we can confirm this finding. We further chose proteins from various stages of the secretory pathway, namely Golgi apparatus (GA) proteins, ER proteins and SNAREs located on GA and ER followed their impact on replication (Fig. 4. 1).
After taking stock of the above-mentioned results on the efficiency of *L. pneumophila* replication after down-regulation of specific trafficking proteins we developed a possible theory. It is securely documented that the ER is hijacked by the LCV. But our findings suggest that Golgi apparatus structure and vesicular trafficking - to and inside the Golgi apparatus - is also important for the pathogen's replication. Figure 3. 12 shows a 50 % - 80 % down-regulation of bacterial burden in cells lacking the structural Golgi apparatus proteins: Golgin84 (20, 9 % replication), Golgin160 (20 % replication) and Giantin (22, 6 % replication). Down-regulation of these proteins is reported to result

in fragmentation of the Golgi apparatus (Heuer *et al.* 2009 and Heuer unpublished). Fragmentation has also been observed after knock-down of Arf1, Rab1 and p115. This severe effect indicates that the scattered Golgi apparatus might negatively influences *L. pneumophila* replication. Absence or at least diminished expression of the integral/ peripheral Golgi apparatus proteins GosR1 (50, 9 % replication) also impaired the pathogens intracellular growth. These proteins are employed in *intra*-Golgi apparatus trafficking, exhibiting interaction sites and tethering functions. Tethers like p115 (52, 8 % replication) and SNAREs like Stx5 (22, 8 % replication) that interact with Rab1 (10 % replication) were definitely also needed for *L. pneumophila* replication. The proteins CASP (a tether corresponding to Golgin84) and NSF (needed for dissociation of ternary SNARE complexes) gave varying results. One time their knock-down diminished growth, one time it even enhanced *L. pneumophila* replication. With one Golgi apparatus structure protein, GM130 though it does not lead to Golgi fragmentation after knock-down - here too *L. pneumophila* showed diminished growth. These results need to be further investigated.

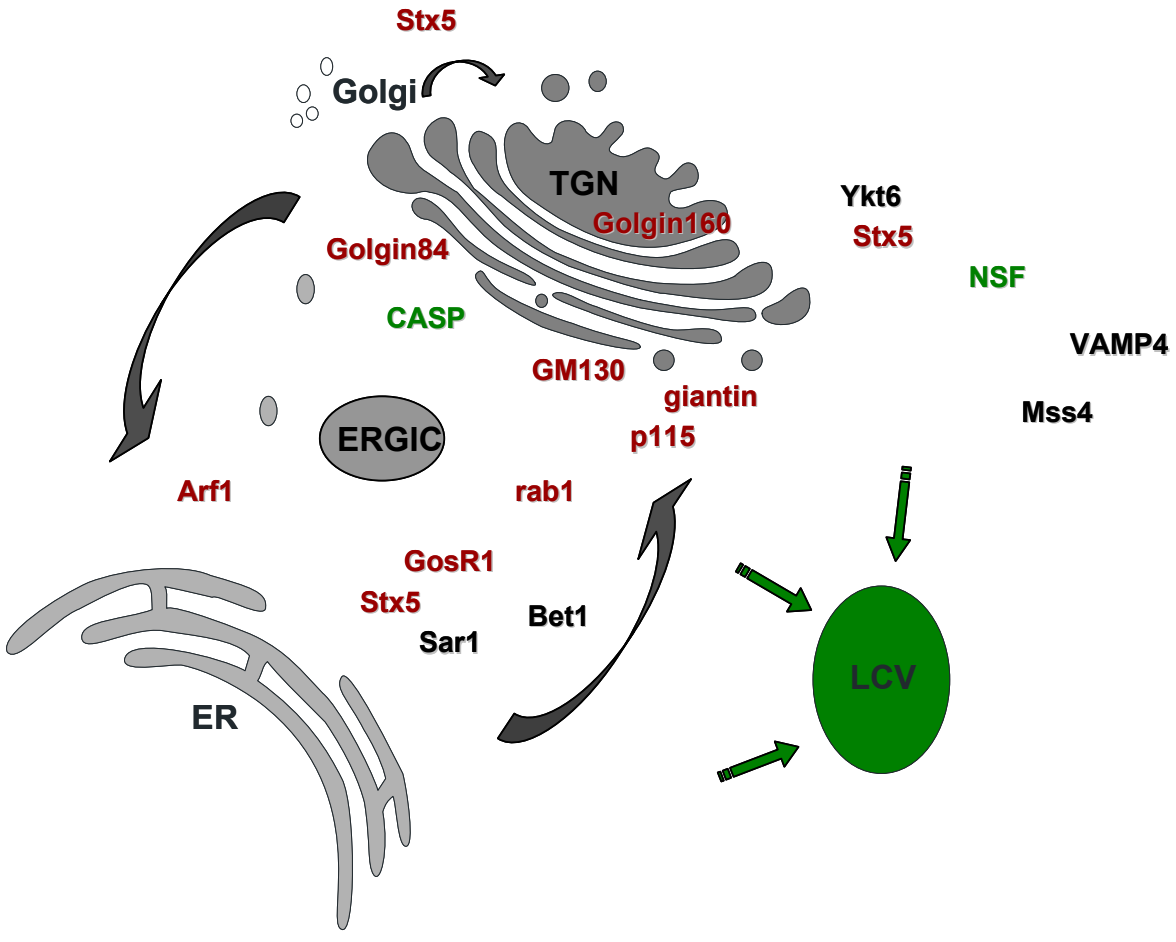

Fig. 4. 1. Model of host protein trafficking in a *L. pneumophila* infected cell.
Anterograde and retrograde trafficking pathways and the distribution of their specific proteins. Red proteins were found to be important for *L. pneumophila* replication; their absence resulted in less replication. Black proteins did not influence *L. pneumophila* growth. Green proteins had varying effects within the assay, their absence diminished growth or enhanced it at different points in time. The green egg represents the *L. pneumophila* containing vacuole.

Since the modulation of organelle trafficking events seems to be of some importance during intracellular multiplication, we looked for *L. pneumophila* effectors that resemble components of the SNARE system (Söllner *et al.* 2002) disabling or altering its function. The *L. pneumophila* proteins LepA and B are shown to have limited sequence similarity to mammalian proteins involved in vesicle trafficking, the so-called SNAREs (Chen *et al.* 2004). Another effector LidA is known to interact with *L. pneumophila* co-effector SidM (DrrA) in vesicle interception. SidM (DrrA) localises to the cytosolic face of the LCV and recruits and regulates Rab1's activity at the LCV (Machner & Isberg 2006, Murata *et al* 2006). SidM and LidA are involved in recruitment of early secretory vesicles during biogenesis of the *L. pneumophila*-containing vacuole (LCV) by mediating recruitment of Rab1 onto *L. pneumophila*-containing vacuoles and in maintaining integrity of the Dot/ Icm complex (Derré *et al.* 2005, Conover *et al.* 2003, Machner & Isberg 2007). The LidA protein exhibits properties important for maintenance of bacterial cell integrity: it associates with the phagosomal surface, promotes replication vacuole formation, and is important for intracellular growth (Conover *et al.* 2003). Loss of LidA does not cause as severe a defect in intracellular growth as exhibited by the *ΔdotA* strain (Conover *et al.* 2003) indicating that the function of the protein may be partially redundant. Since LidA is found to bind Rab1, Rab6 and Rab8, the protein may allow *L. pneumophila* to simultaneously target a subset of Rab GTPases involved in different membrane trafficking routes (Machner & Isberg 2006). It is well known now that Rab1A/ Rab1B and Rab2 are involved in membrane docking in ER to Golgi-apparatus vesicular trafficking (Nuoffer *et al.* 1994, Wilson 1994, Tisdale *et al.* 1992 and Kagan *et al.* 2004) in combination with at least one SNARE complex composed of one v-SNARE (Sec22b) and three cognate t- SNAREs (Stx5, membrin, and Bet1) (Hay *et al.* 1997, Xu *et al.* 2000).

Here we propose a mechanism of *L. pneumophila´s* LidA similar to *Chlamydia´s* IncA. IncA is an effector in chlamydial infection that has been modelled in parallel four helix bundles based on the structure of the SNARE complex. From this model it has been proposed that these proteins may have co-evolved with the SNARE machinery for a role in membrane fusion (Delevoye *et al.* 2004). SNARE´s coiled-coil domains enable interaction with other protein's coiled-coil domains for example membrane bound proteins to bind and help fusion of lipid vesicles with the membrane or other targets. In eukaryotic cells, the fusion of compartments is preceded by assembly of the fusion machinery, including, tethers, GTP-bound proteins and SNAREs. The hallmark of all SNARE proteins is that they contain coiled-coil structures with a highly conserved glutamine or arginine residue at the centre of the complex. This position brings opposite membranes together during SNARE complex formation and is essential for membrane fusion. SNAREs are commonly divided into Q- and R-SNAREs, depending on the central amino acid of this complex being either glutamine (Q) or arginine (R). The heterotrimeric synaptic fusion complex of the SNAREs was proposed by Rothman & Warren in 1994 the so called "zipper-model" (see also: Fasshauer *et al.* in 1998) in which fusion-competent SNARE complexes generally consist of four-helix bundles composed of three Q-SNAREs and one R-SNARE. Their interaction forms a core which is composed of a polar zero layer (Q or R) and a flanking leucine-zipper layer that acts as a water tight shield to isolate ionic interactions in the zero layer from the surrounding solvent. The results given in this study do fit very well into this theory, for Sec22b as well as Stx5 knock-down had a severe impact on *L. pneumophila* replication. Based on these

observations and on our results we propose that LidA may have co-evolved with the SNARE machinery for a role in membrane fusion. This can be deduced from an alignment of the coiled-coil regions of LidA with the defined SNARE regions of Stx5, Membrin, Bet1 and Sec22B. Absence of Stx5 results in severely diminished *L. pneumophila* replication. Modelling *in silico* suggests the possible interaction of LidA as a SNARE.

The role of Golgi apparatus and its structure

Many of the proteins that give structure to the Golgi apparatus as well as proteins trafficking at the *cis*-Golgi apparatus seem to be important in *L. pneumophila* infection; The TGN-proteins as such do not influence replication in a decisive manner. Most of the Golgi apparatus proteins investigated have coiled-coil structures that either anchor them to the membrane and/ or enable them to interact with other proteins, mostly in the manner of SNAREs or tethers. We showed that *L. pneumophila* replicated less in cells with fragmented Golgi apparatus via knock-down of Golgin84, Golgin160, Giantin, Rab1, Arf1 and p115 (Golgi fragmentation experiments by Heuer unpublished). Our model suggests that the dispersed Golgi apparatus interferes severely with *L. pneumophila* replication, most probably by hindering the phagosome to become a vacuole. One possible explanation is that the LCV mimics the Golgi apparatus, to intercept vesicular trafficking from the ER. A dispersed Golgi apparatus, in so-called Golgi apparatus-mini-stacks has a much bigger surface than an intact Golgi apparatus. It becomes a competitor to the LCV, since it provides more docking sites for the ER derived vesicles than the LCV. Thus the *L. pneumophila* strategy of hijacking vesicles by effectors placed on its LCV-surface is rendered ineffective followed by a slowed-down phagosome-construction and the disability to replicate successfully (Fig. 4. 2). We should like to postulate a model that underlines the necessity of an intact Golgi structure in *L. pneumophila* infection, in sharp contrast to the observations of Heuer *et al.* 2009 that show a fragmented Golgi apparatus to boost replication of *Chlamydia*.

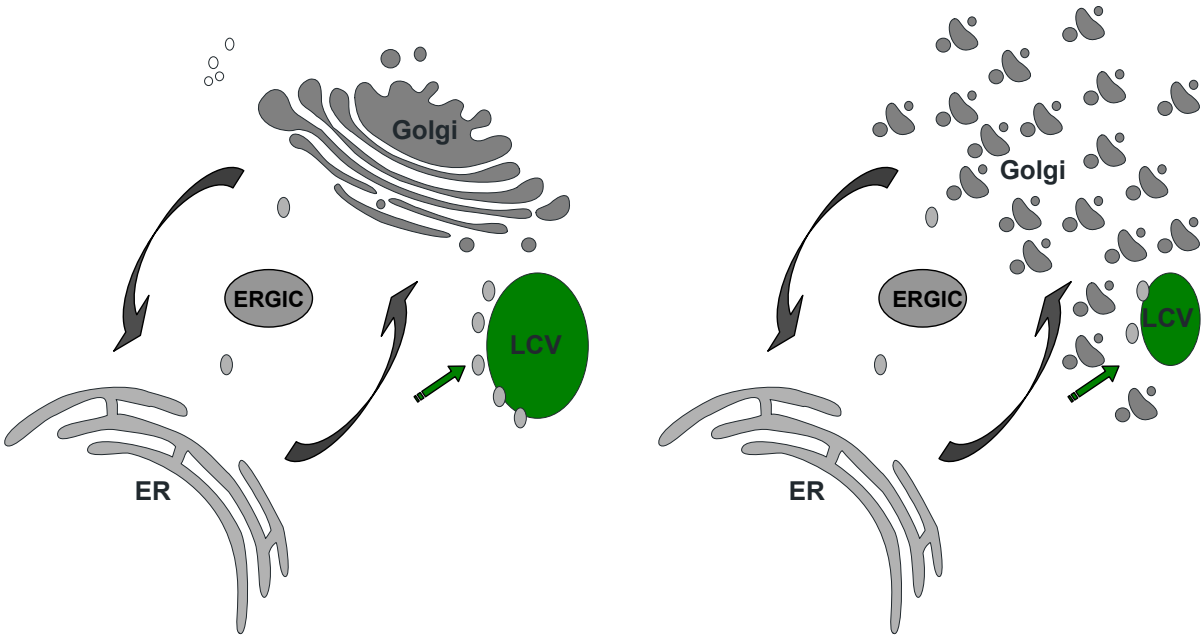

Fig. 4. 2. Resulting model of the influence of Golgi apparatus fragmentation on *L. pneumophila*.
(Left) In intact cells the LCV hijacks vesicles coming from the anterograde pathway, trafficking to the Golgi apparatus by mimicking the Golgi apparatus through exhibiting Golgi apparatus-like proteins on its surface. (Right) In a cell where the Golgi apparatus is artificially fragmented only leaving it with Golgi apparatus mini-stacks, the surface of the Golgi apparatus is very much enlarged. This diminishes vesicle-LCV interaction events compared to vesicle-Golgi apparatus interaction events. The LCV is starved off vesicles and can not efficiently construct its replication niche. *L. pneumophila* replicates less in cells with fragmented Golgi apparatus.

The above postulated model does exhibit two flaws. First, the cross-talk of *L. pneumophila* with the host-ER is well known. Here we could show that even the ER-Golgi apparatus intermediate compartment (ERGIC) is attached to the phagosome. But we could not verify in our studies that Sar1 knock-down has any impact on *L. pneumophila* replication though it is an important protein in ER to Golgi apparatus transport. Sar1 similar to Arf1 assists in COP vesicle formation at the ER directed to the Golgi apparatus. The knock-down of Sar1 in this work was verified only on mRNA level, but the actual amount of protein was not tested. So the observed effect might be due to the fact, that the protein level was not impaired. Second, also this model does not explain the effect of GM130. Down-regulation of GM130 does not affect Golgi structure (Heuer *et al.* 2009) but has a negative effect on *L. pneumophila* replication. The impact of the GM130 interaction partners Rab1 and Stx5 on the pathogen´s replication is almost comparable. Co-localisation of GM130 with the phagosome does seem to somehow connect the phagosome to the Golgi apparatus. Ectopic expression of SidM, the *L. pneumophila* effector that specifically interacts with Rab1 is shown to interfere with ER-to-Golgi apparatus trafficking and induces the dispersal of the Golgi apparatus (Machner & Isberg 2006, Murata *et al* 2006). Since GM130 is one of the interaction partners of Rab1, regulating vesicular transport, its absence might devoid *L. pneumophila´s* effectors of an important communication protein interacting with Rab1 (f. e. activating) and by this only just enabling the effectors, to recruit Rab1. How exactly this is achieved is a matter of further investigation. The exact role of Stx5 has to

be further investigated. The phagosome could for example recognise the SNARE residue of Stx5 maybe by use of effectors and thereby employ it for its own needs.

The depicted model draws a whole new picture of *L. pneumophila* interaction with its host cell on the cellular level as well as in comparison to other intracellular pathogens such as *Chlamydia* and *Salmonella*. These two intracellular pathogens are known for their specific interaction with the Golgi apparatus. *Chlamydia* for example preys on a fragmented Golgi apparatus. Its growth is enhanced by an artificially scattered Golgi structure (Heuer *et al.* 2009). This was also connected to a physical need, for the *Chlamydia* containing inclusion in epithelial cells is enormous, compared to the cell and does not leave much space for the resident organelles. *Salmonella* are also known to interact with the Golgi apparatus, but their exact course of action is not yet understood. All of the results from the *L. pneumophila* mini-screen viewed in combination with the *in silico* investigation of the *L. pneumophila* LidA effector interaction with host-cell trafficking proteins and the visible co-localisation of the ER and Golgi apparatus proteins with the *L. pneumophila* phagosome underline the multiple ways of cross talk of the pathogen and its host in the secretory pathway. After particularly investigating the role of Golgi apparatus proteins: matrix proteins, vesicle SNAREs and tethers localised all over the Golgi apparatus it did not escape our attention that this organelle plays a significant role in *L. pneumophila* infection.

4. 3 Protein phosphorylation on host proteins upon *L. pneumophila* infection

Bacterial infection of eukaryotic cells alters host cell protein functions. Some pathogenic bacteria have evolved clever survival strategies for manipulating host cell signalling pathways to establish beneficial replicative niches within the host. Protein phosphorylation is one example of a broad range of tools, by which effectors address key host proteins to remodel signalling events targeting a wide range of effects. Phosphorylation defines protein fate by providing a reversible way to regulate protein–protein interactions both spatially and temporally. Addition of a phosphoryl to a serine, threonine or tyrosine residue of a protein has certain effects. It for example enables interaction with specific "recognition domains". In some proteins phosphorylation creates binding sites for modular phospho-protein-binding domains, which connect these molecules with their upstream kinases and downstream effectors to form multi-protein complexes that monitor their activity, binding partners or cellular localization. Phosphorylation might also define the thermodynamics of energy-requiring reactions significantly. In addition enzyme inhibition or even protein degradation is mediates on this way. The idea that specific domains bind to distinct phosphorylated sequence motifs enlarges the possibilities of the functions of cellular proteins in breadth and diversity.
In *L. pneumophila* infection virulence factors, especially the effectors translocated into the host cytosol have been shown to specifically interact with host proteins. Up until now we know of at least two specific ways by which *L. pneumophila* effectors interact with their host on basis of phosphorylation activities. One is the manipulation of the Rab-GTPase function by RalF. This effector functions as an activator of the small GTPase Arf1, involved in retrograde vesicle transport from the Golgi apparatus compartment to the ER to *L. pneumophila*-containing phagosomes, by enabling

exchange of GDP for GTP (Nagai *et al.* 2002). The second modulation is carried out by SidC, binding to phospho-inositide (PI (4) P) on the vacuole (Luo & Isberg 2007)). This anchoring to the LCV directly engages host cell components and subverts vesicle trafficking or functions as membrane anchor/ adaptor-proteins for other Dot/ Icm-secreted effector proteins.

In this study we wanted to obtain a global overview over host protein phosphorylation upon *L. pneumophila* infection. The following project was based on observations by Coxon *et al*. 1998 and Susa *et al.* 1999 showing induction of tyrosine protein phosphorylation in MRC-5 cells after *L. pneumophila* invasion 24 hours post infection, as well as activation of tyrosine kinase, protein kinase C and induction of actin polymerisation in human monocytes. To make sure to obtain a global overview, we in this case used *Acanthamoeba castelanii* as a model for infection, since several intracellular pathogens including *L. pneumophila* but also *Chlamydia spp.* (Essig *et al.* 1997) are able to replicate within protozoa and human cells. From this it has been postulated that free-living amoebae may serve as a reservoir for pathogens in the environment, perhaps even as a "training environment" for the selection of virulence-related traits in these pathogens (Gao *et al.* 1997). Under experimental conditions, *L. pneumophila* are known to multiply within and kill a variety of phylogenetically unrelated protozoa ranging from *Acanthamoeba castellanii* to the genetically well characterised social amoeba *Dictyostelium discoideum* (Rowbotham *et al.* 1986). Under the assumption that amoeba provide a possible training ground we went back to *L. pneumophila´s* natural host as model of infection. *Acanthamoeba castellanii* should definitely show a phosphorylation pattern after infection with wt *L. pneumophila*.

This setting resulted in the detection of a 44 kDa protein kinase, phosphorylated after *L. pneumophila* infection. The protein is phosphorylated at a tyrosine residue, since we specifically searched for phospho-tyrosine sites using a specific antibody. The peptide sequence gained from *Acanthamoeba* is annotated to a kinase involved in the general signalling pathways of the cell. The ser-thr-protein kinase with a catalytic domain at the tyrosine residue is a phospho-transferase. The enzymatic activity of these protein kinases is controlled by phosphorylation of specific residues in the activation segment of the catalytic domain. Here the hit tyrosine site matches the theory well.
A blast search of the peptide given from the LC MS/MS spectrum on the human genome resulted in a protein sequence with homology to glycogen synthase kinase 3 (GSK3). This GSK3 also contains the tyrosine, but a slightly different sequence of the peptide, starting with E instead of S. GSK3 is a proline-directed serine-threonine kinase that was initially identified as a phosphorylating and inactivating glycogen synthase. GSK3 is involved in energy metabolism, neuronal cell development, and body pattern formation (Plyte *et al.* 1992). This kinase phosphorylates and inactivates the glycogen-synthase connected to the wnt-signalling cascade through β-catenin. It is itself regulated by phosphorylation via the protein kinase B. In other cases phosphorylation is sometimes combined with reversible conformational changes. Activation at this point would enable further phosphorylation within a cascade of proteins. This hints to a large impact of the pathogen in all different cellular information pathways, opening up the door for a whole new field of interest. Due to

lack of time these experiments were not pursued. But it would definitely be promising to shed light on signalling pathways especially on the ones of high information load as tyrosine phosphorylation.

4. 4 NF-κB response upon *L. pneumophila* infection

Manipulation of host signalling is crucial to promote pathogen survival. Bacteria's ability to replicate depends on their survival within the cell as well as the survival of the cell until bacterial replication is completed. One factor in promoting anti-apoptosis is the mammalian nuclear transcription factor-κB (NF-κB). NF-κB describes a whole family of transcription factors that link extracellular stimuli to cellular responses and thereby control for example inflammation, innate immunity, cell division, and survival (Karin & Greten 2005, Hoffmann & Baltimore 2006, Hayden & Ghosh 2008). Activation of the NF-κB-signalling cascade induces dissociation of the dimeric transcription factors p65/ RelA and p50 (in the case of the classical pathway). In inactivated cells these dimeric factors are constantly bound to their inhibitor IKK. This complex is thought to oscillate from the cytosol into the nucleus and back again. When IKK is phosphorylated, the bound factors are released. These factors then translocate to the nucleus permanently, where they bind DNA and enable transcription of defined regions leading to a concerted anti-apoptotic response. In cancer cells p65 stays inside the nucleus. This might be caused by a variety of mechanisms such as constitutive stimulation of IKK, mutations or amplifications in NF-κB genes or mutations in inhibitors of NF-κB. Since NF-κB regulates the transcription of several anti-apoptotic genes and also stimulates proliferation, it has been suggested that the constitutive activation of NF-κB confers resistance to apoptosis and in some cases may stimulate growth.

In Bartfeld *et al.* 2009 we were able to show that *L. pneumophila* infection activates NF-κB in a biphasic activation pattern: Losick and Isberg demonstrated an influence of the multiplicity of infection (MOI) on NF-κB activation (Losick & Isberg, 2006): low dose infections (MOI 1) led to Dot/ Icm-dependent and MyD88-independent NF-κB activation, whereas at an MOI 10 signaling occurred via MyD88 and in a Dot/ Icm-independent manner. The reason for this observation was not known. In our work we were able to attach the MOI-dependant differet activation-patterns to two different phases in time. The first transient activation is mediated by flagellin via the TLR5 and MyD88 pathway and is soon silenced. The second activation leads to permanent nuclear translocation of p65 that depends on the replication of bacteria and a functional Dot/ Icm system. A higher MOI would entail a bacterial invasion of the cell and activation of NF-κB by the Dot/ Icm system. A lower infectious dose would only result in a TLR5/MyD88-triggered IL8 activation. The specific activation of NF-κB in *L. pneumophila* infection depending on its type IV secretion system leads to survival of the cell although the *L. pneumophila* containing vacuole is filled with replicating bacteria. The unusual pattern of continuous nuclear localization of p65 over hours without oscillation may hint at an active interference of the bacteria with the NF-κB system. Since the Dot/ Icm system is crucial for this permanent activation, it seem logical to propose one or more of the type IV-dependent effectors to be involved in activation of the NF-κB regulated pathway. It is assumed that the transcription factor p65 remains inside the nucleus, thereby permanently activating the anti-apoptotic signalling. The

anti-apoptotic effects of NF-κB might particularly be beneficial for intracellular pathogens that depend on the survival of their hosts. Therefore, it seems plausible that *L. pneumophila* is able to actively manipulate NF-κB. From the NF-κB activation assay (described in 3. 4) and subsequent analyses employing *L. pneumophila* mutants, two mutants defective in Dot/ Icm effectors giving an unusual activation pattern attracted attention. One *ΔsdbA* deletion mutant is no longer able to induce the permanent p65 translocation although the flagellin-dependent transient activation still is detected. Another mutant lacking *sdhA* shows normal NF-κB activation although. SdhA is described to be deficient in activation of anti-apoptotic signals (Laguna *et al*. 2006). Two distinct *L. pneumophila* effectors are already known to specifically inhibit macrophage cell death. One of these is the described SdhA, the other is SidF (Laguna *et al.* 2006, Banga *et al.* 2007). While SidF directly interacts with and neutralises the activity of pro-death members of the Bcl-2-family, the mechanism employed by SdhA is not known. Here we could show that deletion of *sdhA* targets the infected cells to apoptosis and decreases bacterial intracellular growth.

Our results show that the Philadelphia*ΔsdhA* mutant is a slightly diminished in replication within A549 cells – compared to the Philadelphia wt. Replication of the Philadelphia*ΔdotA* mutant even decreased, as usual, because of *L. pneumophila* exiting the cell, being killed in the Gentamicin containing medium (this has been discussed at the beginning of this chapter). These findings are corroborative of the results of this mutant's replication being reduced in mouse bone marrow derived macrophages (Laguna *et al.* 2006). In this study we are able to demonstrate that this also holds true for human epithelial cells. Though not much is known about SdhA, the effector SdbA has been even less investigated. The mutant lacking *sdbA* did show even fewer replication in human A549 cells than *ΔsdhA*. The bacterial burden of Philadelphia*ΔsdbA* in epithelial cells remained constant in comparison to a slight decrease in the Philadelphia*ΔdotA* mutant's burden, but it did not grow. Since the mutant lacking the complete Dot/ Icm system again was killed after exiting the cell; *ΔsdbA* must have either replicated a little or might even give some anti-apoptotic signal, because the bacterial burden at least did not grow less. The inability for intracellular growth mirrors their complete lack of p65 activation. The fact that the deletion of one single effector has such a severe impact is remarkable due to the high redundancy of the *L. pneumophila* effector system, meaning deletion of a single effector rarely has such a big impact on replication. This renders SdbA an especially interesting effector.

The SdbA protein and its function are not yet well characterised, although we know that it contains 3354 bp (giving 1117 aa) and that it is a putative substrate of the Dot/ Icm system with a calculated molecular mass of 127075 Da. We show here that the secreted protein SdbA is important for intracellular growth. The *sdbA* mutant, as well as other mutants tested, known for defects in intracellular replication, does not lead to permanent NF-κB activation (Bartfeld *et al.* 2009). Our data does not allow conclusions on whether SdbA has a direct influence on NF-κB signaling or not. Growth restriction of *ΔsdbA* was also observed in bone marrow derived macrophages from A/ J mice (Luo & Isberg 2004). We can only confirm a tight connection of intracellular growth and permanent p65

translocation. Future experiments need to elucidate this connection between replication; Dot/ Icm secreted factors and permanent NF-κB activation. To shed light on this connection we started experiments to over-express SdbA in eukaryotic cell, planning to use these on a microarray, but this project could not be completed due to lack of time.

Conclusion

Aim of this study was to find out, how *L. pneumophila* interacts with its host cell to ensure its own survival and replication. We answered this question by successfully setting up a *L. pneumophila*-infection-assay in combination with siRNA-mediated manipulation of specific host cell proteins. These two methods channelled into the investigation of the impact of several Golgi apparatus and ER proteins, vesicular SNAREs and tethers particularly in *L. pneumophila* replication. Especially intriguing was the detection of a set of Golgi structure proteins, whose function in *L. pneumophila* infection has until now not been analysed. From this we hypothesise a new model of the *Legionella* containing vacuole mimicking the Golgi apparatus, through proteins presented on its surface. Adding to this theory we could show that the *L. pneumophila* effector LidA displays a putative SNARE-motif enabling for interaction with the host SNARE-machinery. This might contribute to successful hijacking of vesicles from the anterograde trafficking pathway. This model we deduce from the fact that *L. pneumophila* shows decreased replication in cells with a fragmented Golgi structure upon siRNA knock-down of Golgi structure proteins. In these cells the Golgi apparatus is reduced to mini stacks, presenting a larger surface than the intact Golgi apparatus. Here vesicles coming from the ER primarily meet the Golgi apparatus instead of the Legionella containing vacuole, so that the vacuole is delayed in construction. A distinct interaction should be further investigated.

One still lingering question was the mechanism via which the pathogen interacts with its host cell. The knowledge of the importance of phosphorylated and dephosphorylated proteins lead us to specifically search for modulated host cell proteins after *L. pneumophila* infection. Here we used an approach to find phosphorylated tyrosine sites in the proteome of the *L. pneumophila* infected natural host *Acanthamoeba castellanii*. The ability of host-pathogen cross-talk via phosphorylation has in the past for example been connected to sub-cellular activities such as cytoskeleton rearrangement and signalling events. We were able to detect a 44 kDa serine-threonine- kinase with a tyrosine phosphorylation site that is very much enhanced in infected *Acanthamoeba* compared to uninfected amoeba. The human homologue for this protein is a GSK3 kinase involved for example in wnt-signalling. These first initial results open the door for a whole new field of research.

Knowing that *L. pneumophila* not only interacts with signalling of its host secretory pathways but also by inhibiting apoptosis, the last question covered in this work involves the reported bi-phasic activation of NF-κB post infection. It was aimed to further clarify which *L. pneumophila* effectors might be involved in this specific signalling event. Two of these effectors have been investigated: SdbA and SdhA. Whereas deletion of SdhA only slightly diminished *L. pneumophila* replication, SdbA resulted in a complete loss of growth in epithelial cells, confirming a tight connection of intracellular growth and permanent p65 translocation. Further experiments including eukaryotic SdbA over expression succeeded by micro-array analysis should be pursued.

Abbreviation	Description
BCL-XL	Basal cell lymphoma-extra large
BLAST	Basic Local Alignment Search Tool
BSA	Bovine serum albumin
COX2	Cyclooxygenase
cRel	reticulo-endotheliosis viral oncogene homolog B
DMEM	Dulbecco´s modified eagle medium
DMSO	Dimethyldulfofid
DNA	Desoxyribonucleic acid
Dot/ Icm	Defective for organelle trafficking/ intracellular multiplication
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ERGIC	ER–Golgi intermediate compartment
FCS	Fetal calf serum
FITC	Flouresceinisothiocyanat
GA	Golgi apparatus
GAP	GTPase activating protein
GDP	Guanosindiphosphat
GFP	Green fluorescent protein
GSK3	Glycogen synthase kinase 3
GTP	Guanosintriphosphate
h	Hour
HL60	Human promyelocytic leukemia cells
HSP60	Heat shock protein 60
IL-6	Interleukin 1
IL-8	Interleukin 8
kDa	Kilo dalton
L. bozemanii	*Legionella bozemanii*
L. longbeachae	*Legionella longbeachae*
L. micdadei	*Legionella micdadei*
L. pneumophila	*Legionella pneumophila*
LAMP	Lysosomal associated membrane protein
LCV	*Legionella* containing vacuole
MHC I / MHC II	Major histocompatibility complex
MOI	Multiplicity of infection
MyD88	Myeloid differentiation primary response gene88
NES	Nuclear export sequences
NF-κB	Nuclear factor κB
NLS	Nuclear localization sequence
PBS	Phosphate buffered saline

Abbreviation	Description
PFA	Paraformaldehyd
PI3K	Phosphotidyl-inositol 3 kinase
PK	Tyrosine kinase
PKA	Protein kinase A
PKC	Protein kinase C
P-Tyr	Phospho-tyrosine
RelA	Reticulo-endotheliosis viral oncogene homolog A
RISC	RNA induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA interference
rpm	Rounds per minute
RT	Room temperature
RV	Replication vacuole
s	Seconds
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel-electrophoresis
SH2	Src homology 2 domain
siRNA	Small interfering RNA
src	sarcoma
TEMED	N, N, N´, N´-Tetramethylendiamin
TLR-5	Toll-like receptor
TNF	Tumor-necrosis factor
Tris	Tris-(hydroxymethyl)-Aminomethan
type IV	Type IV secretion system
VBNC	Viable but not culturable
w/ v	Weight per volume
Wnt	merged from the names of the homologues genes Wg (*Drosophila melanogaster*) and Int (mouse) both employed in evolutionary developement
wt	Wild type

Acknowledgements

My sincere thanks go to Prof. Dr. Thomas F. Meyer for providing the opportunity to work in the exceptional conditions at the Department of Molecular Biology and for guidance on my thesis work.

Many thanks to PD Dr. Antje Flieger for supervising this thesis and providing great help in any *Legionella* related question.

In particular, I want to thank Dr. Dagmar Heuer and Dr. Holger Brüggemann for initiating the various projects and for their scientific advice. Their guidance and patience to explain and to discuss the experimental work were of invaluable help throughout this project. The suggestion to use the NF-κB activation screen in *L. pneumophila* infection and the set-up as well as the leading experiments derive from Sina Bartfeld, and I am very grateful for her help and enthusiasm about my work.

Most of all I want to thank my lab- and officemates, especially Malvika Pompaiah, Elena Belogolova, Sebastian Banhart, Sabrina Janssen, Carsten Holland and Oliver Riede for a great working atmosphere, for all the help and guidance night or day concerning any possible and impossible problems. Thanks to Charlotte Meissner and Anja Greiser without whom I would not have survived.

I am indebted to many people, who supported me: Monika Schmitt and Peter Jungblut (Protein-Analysis Core Facility, MPIIB) performed the nanoflow liquid chromatography tandem mass spectrometry. Nikolaus Machuy, Chris Dimmler, David Mantz and Dominique Kahlil (RNAi Core Facility, MPIIB) provided me with siRNA and the needed equipment as well as Volker Brinkmann (Microscopy Core Facility, MPIIB) who instructed me on the confocal microscope. Maja and Bastian (Charité Campus Virchow, Berlin) introduced me to Legionella - thank you very much. Dr. Philipp Auraß and Enrico Siegbrecht (RKI, Berlin) lead me further in my work concerning Legionella.

Many thanks go to Dr. Jura Churin, Dr. Hiroshi Asakura, Dr. Lina Fassi-Fehri, Dr. Simone Hess, Dr. Andre Maeurer, Dr. Mirjana Kessler and Dr. Daniel Becker (Department of Molecular Biology, MBIIB) for the help and support through the past years. And thanks to Dr. Lesley Ogilive for reading and revising this work.

Furthermore I am much obliged to Dagmar Frahm, Elke Ziska, Kirstin Hoffmann, Jörg Angermann, Susan Jackisch, Meike Soerensen as well as Angela and Angela (Department of Molecular Biology, MBIIB) for providing the basis for any work done in the department by contributing knowledge, cells, enzymes and lots of agar-plates and supplements. Many thanks also go to Kerstin Rydzewsky (RKI) for her helpful technical support and advice.

Finally I am most grateful to my family and my „Berlin family“ for love and support in tough times.

Publications

Lafi, F. F., Fuerst, J. A., Fieseler, L., Engels, C., Goh, W. W., Hentschel U. (2009). Widespread distribution of poribacteria in demospongiae. *AEM*

Bartfeld, S., Engels, C., Bauer, B., Aurass, P., Flieger, A., Brüggemann, H. & Meyer, T. F. (2009). Temporal resolution of two tracked NF-κB activation by *Legionella pneumophila*. *Cell. Microbiol.*

Engels, C., Heuer, D., Rejmann-Lipinski, A., Brüggemann, H., Flieger, A. & Meyer, T. F. (2010). Involvement of Golgi structure proteins in *Legionella pneumophila* replication. (in preparation)

Selbständigkeitserklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig und nur unter Verwendung der angegebenen Hilfsmittel angefertigt habe.

Berlin, 28. April 2009

Literature

Abu Kwaik, Y. & Engleberg, N. C. (1994). Cloning and molecular characterization of a *Legionella pneumophila* gene induced by intracellular infection and by various *in vitro* stress conditions. *Mol Mic* **13**, 243-251.

Abu Kwaik, Y. & Pederson, L. L. (1996). The use of differential display-PCR to isolate and characterize a *Legionella pneumophila* locus induced during the intracellular infection of macrophages. *Mol Microbiol* **21**, 543-556.

Abu Kwaik, Y. (1996). The phagosome containing *Legionella pneumophila* within the protozoan *Hartmannella vermiformis* is surrounded by the rough endoplasmic reticulum. *Appl Environ Microbiol* **62**, 2022-2028.

Abu Kwaik, Y. (1998b). Fatal attraction of mammalian cells to *Legionella pneumophila*. *Mol Microbiol* **30**, 689-695.

Abu Kwaik, Y. (1998d). Induced expression of the *Legionella pneumophila* gene encoding a 20- kilodalton protein during intracellular infection. *Infect Immun* **66**, 203-212.

Abu Kwaik, Y. (2000). Invasion of Mammalian and Protozoan Cells by *Legionella pneumophila*. In *Bacterial Invasion into Eukaryotic Cells*. Edited by T. A. O. a. J. Hacker. *New York, Boston, Dordrecht, London, Moscow: Kluwer Academic / Plenum Publishers*.:383-410.

Abu Kwaik, Y., Fields, B. S. & Engleberg, N. C. (1994). Protein expression by the protozoan *Hartmannella vermiformis* upon contact with its bacterial parasite *Legionella pneumophila*. *Infect Immun* **62**, 1860-1866.

Abu Kwaik, Y., Gao, L. Y., Harb, O. S. & Stone, B. J. (1997). Transcriptional regulation of the macrophage-induced gene (*gsp*A) of *Legionella pneumophila* and phenotypic characterization of a null mutant. *Mol Microbiol* **24**, 629-642.

Abu Kwaik, Y., Gao, L. Y., Stone, B. J., Venkataraman, C. & Harb, O. S. (1998a). Invasion of protozoa by *Legionella pneumophila* and its role in bacterial ecology and pathogenesis. *Appl Environ Microbiol* **64**, 3127-3133.

Abu Kwaik, Y., Venkataraman, C., Harb, O. S. & Gao, L. Y. (1998c). Signal transduction in the protozoan host *Hartmannella vermiformis* upon attachment and invasion by *Legionella micdadei. Appl Environ Microbiol* **64**, 3134-3139.

Abu-Zant, A., Jones, S., Asare, R., Suttles, J., Price, C., Graham, J. & Abu Kwaik, Y. (2007). Anti-apoptotic signalling by the Dot/Icm secretion system of *L. pneumophila*. *Cell Microbiol* **9 (1),** 246–264.

Albert-Weissenberger, C., Cazalet, C. & Buchrieser, C. (2007). *Legionella pneumophila* - a human pathogen that co-evolved with fresh water protozoa. *Cell. Mol. Life Sci.* **64,** 432–448.

Al-Khodor, S., Price, C. T., Habyarimana, F., Kalia, A. & Abu Kwaik, Y. (2008). A Dot/Icm-translocated ankyrin protein of *Legionella pneumophila* is required for intracellular proliferation within human macrophages and protozoa. *Mol Mic* **70 (4),** 908–923.

Allan, V. J., Thompson, H. M. & McNiven, M. A. (2002). Motoring around the Golgi apparatus. *Nat Cell Biol* **4,** E236-E242.

Allan, B. B., Moyer, B. D. & Balch, W. E. (2000). Rab1 recruitment of p115 into *cis*-SNARE complex: programming budding COPII vesicles for fusion. *Science* **289,** 444–448.

Amer, A. O., & Swanson, M. S. (2005). Autophagy is an immediate macrophage response to *Legionella pneumophila*. *Cell. Microbiol.* **7,** 765–778.

Aoe, T., Cukierman, E., Lee, A., Cassel, D., Peters, P. J. & Hsu, V. W. (1997). The KDEL receptor, ERD2, regulates intracellular traffic by recruiting a GTPase-activating protein for ARF1. *EMBO J* **16**, 7305-7316.

Aragon, V., Kurtz, S., Flieger, A., Neumeister, B. & Cianciotto, N. P. (2000). Secreted enzymatic activities of wild-type and *pil*D-deficient *Legionella pneumophila*. *Infect Immun* **68**, 1855-1863.

Archer, K. A., Alexopoulou, L., Flavell, R. A. & Roy, C. R. (2008). Multiple MyD88-dependent responses contribute to pulmonary clearance of *Legionella pneumophila*. *Cell Microbiol* 1-16.

Arenzana-Seisdedos, F., Thompson, J., Rodriguez, M. S., Bachelerie, F., Thomas, D.,& Hay, R. T. (1995). Inducible Nuclear Expression of Newly Synthesised IκBα Negatively Regulates DNA-Binding and Transcriptional Activities of NF-κB. *Mol and Cell Biol* **15 (5)**, 2689–2696

Aridor, M., Bannykh, S. I., Rowe, T. & Balch, W. E. (1995). Sequential coupling between COPII and COPI vesicle coats in endoplasmic reticulum to Golgi apparatus transport. *J. Cell Biol.* **131,** 875–893.

Arroyo, J., Hurley, M. C., Wolf, M., McClain, M. S., Eisenstein, B. I. & Engleberg, N. C. (1994). Shuttle mutagenesis of *Legionella pneumophila*: identification of a gene associated with host cell cytopathicity. *Infect Immun* **62**, 4075-4080.

Atlas, R. M. (1999). *Legionella*: from environmental habitats to disease pathology, detection and control. *Environ Microbiol* **1**, 283-293.

Baca, O. G. & Paretsky, D. (1983). Q fever and *Coxiella burnetii*: a model for host-parasite interactions. *Microbiol Rev* **47**, 127-149.

Backert, S. & Meyer, T. F. (2006). Type IV secretion systems and their effectors in bacterial pathogenesis. *Curr. Op. in Microbiol.* **9,** 207–217.

Baeuerle, P. A. & Baltimore, D. (1988). IkB: a specific inhibitor of NF-κB transcription factor. *Science* **242 (4878),** 540-546.

Banga, S., Gao, P., Shen, X., Fiscus, V., Zong, W., Chen, L. & Luo, Z. (2007) *Legionella pneumophila* inhibits macrophage apoptosis by targeting pro-death members of the Bcl2 protein family *PNAS* **104 (12)**, 5121–5126.

Barken, D., Wang, C. J., Kearns J., Cheong, R., Hoffmann, A. & Levchenko, A. (2005). Comment on „Oscillations in NF-κB Signaling Control the Dynamics of Gene Expression". *Sience* **308**, 52a.

Barocchi, M. A., Masignani, V. & Rappuoli, R. (2005). Cell entry machines: a common theme in nature? *nrmicro* **10**

Batrukova, M. A., Betin, V. L., Rubtsov, A. M. & Lopina, O. D. (2000). Ankyrin: structure, properties, and functions. *Biochemistry (Mosc)* **65,** 395-408.

Bellinger-Kawahara, C. & Horwitz, M. A. (1990). Complement component C3 fixes selectively to the major outer membrane protein (MOMP) of *Legionella pneumophila* and mediates phagocytosis of liposome-MOMP complexes by human monocytes. *J Exp Med* **172**, 1201-2110.

Berger, K. H. & Isberg, R. R. (1993). Two distinct defects in intracellular growth complemented by a single genetic locus in *Legionella pneumophila*. *Mol Microbiol* **7,** 7-19.

Berger, K. H. & Isberg, R. R. (1994). Intracellular survival by *Legionella*. *Methods Cell Biol* **45**, 247-259.

Blume-Jensen, P. & Hunter, T. (2001). Oncogenic kinase signalling. *Nature* **411,** 355-365.

Bozue, J. A. & Johnson, W. (1996). Interaction of *Legionella pneumophila* with *Acanthamoeba castellanii*: uptake by coiling phagocytosis and inhibition of phagosome-lysosome fusion. *Infect Immun* **64**, 668-673.

Brand, B. C. & J. Hacker (1997). The Biology of *Legionella* Infection. In *Host Response to Intracellular Pathogens*, pp. 291-311. Edited by S. H. E. Kaufman: R.G. Landes Company.
Brand, B. C., Sadosky, A. B. & Shuman, H. A. (1994). The *Legionella pneumophila icm* locus: a set of genes required for intracellular multiplication in human macrophages. *Mol. Microbiol.* **14,** 797–808.

Brüggemann H., Cazalet C. & Buchrieser C. (2006). Adaptation of *Legionella pneumophila* to the host environment: role of protein secretion, effectors and eukaryotic-like proteins. *Current Opinion in Microbiology* **9,** 86–94.

Brumell, J. H. & Scidmore, M. A. (2007). Manipulation of Rab GTPase Function by Intracellular Bacterial Pathogens. *Mic and Mol Biol Rev* 636–652.

Burkhard, P., Stetefeld, J. & Strelkov, S. V. (2001). Coiled coils: a highly versatile protein folding motif. *Trends Cell Biol.* **11,** 82– 88.

Byrne, B. & Swanson, M. S. (1998). Expression of *Legionella pneumophila* virulence traits in response to growth conditions. *Infect Immun* **66**, 3029-3034.

Campodonico, E. M., Chesnel, L., Roy, C. R. (2005). A yeast genetic system for the identification and characterization of substrate proteins transferred into host cells by the *Legionella pneumophila* Dot/Icm system. *Molecular Microbiology* **56 (4),** 918–933.

Cattani, L., Goldoni, P., Castellani, M., Pastoris, L., Sinibaldi, L. & Orsi N. (1997). Bafilomycin A1 and Intracellular Multiplication of *Legionella pneumophila Antibiotic agents and Chemother* **41 (1),** 212–214.

Cazalet, C., Rusniok, C., Brüggemann, H., Zidane, Z., Magnier, A., Ma, L., Tichit, M., Jarraud, S., Bouchier, C., Vandenesch, F., Kunst, F., Etienne, J., Glaser, P. & Buchrieser, C. (2004). Evidence in the Legionella pneumophila genome for exploitation of host cell functions and high genome plasticity. *Nature Gen* **36 (11),** 1165-1173.

Chang, B., Amemura-Maekawa, J., Kura, F., Kawamura, I., Watanabe, H. (2004). Expression of IL-6 and TNF-α in human alveolar epithelial cells is induced by invading, but not by adhering, *Legionella pneumophila. Microbial Pathogenesis* **37,** 295–302.

Chen, J., de Felipe, K. S., Clarke, M., Lu, H., Anderson, O. R., Segal, G. & Shuman, H. A. (2004). *Legionella* effectors that promote nonlytic release from protozoa. *Science* **303,** 1358-1361.

Chien, M., Morozova, I. Shi, S., Sheng, H., Chen, J., Gomez, S. M., Asamani, G., Hill, K., Nuara, J., Feder, M., Rineer, J., Greenberg, J. J., Steshenko, V., Park, S. H., Zhao, B., Teplitskaya, E., Edwards, J. R., Pampou, S., Georghiou, A., Chou, I.-C., Iannuccilli, W., Ulz, M. E., Kim, D. H., Geringer-Sameth, A., Goldsberry, C., Morozov, P., Fischer, S. G., Segal, G., Qu, X., Rzhetsky, A., Zhang, P., Cayanis, E., De Jong, P. J., Ju, J., Kalachikov, S, Shuman, H. A., Russol, J. J. (2004). The Genomic Sequence of the Accidental Pathogen Legionella pneumophila. *Science* **305,** 1966-1968.

Chiu, R., Novikov, L., Mukherjee, S. & Shields, D. (2002). A caspase cleavage fragment of p115 induces fragmentation of the Golgi apparatus and apoptosis. *JCB* **159 (4),** 637-648.

Christie, P. J. & Vogel, J. P. (2000). Bacterial type IV secretion: conjugation systems adapted to deliver effector molecules to host cells. *Trends Microbiol* **8**, 354-360.

Cianciotto, N. P., Stamos, J. K., Kamp, D. W. (1995). Infectivity of *Legionella pneumophila* mip mutant for alveolar epithelial cells. *Curr Microbiol* **30 (4),** 247-250.

Cirillo, J. D., Falkow, S. & Tompkins, L. S. (1994). Growth of *Legionella pneumophila* in Acanthamoeba castellanii enhances invasion. *Infect Immun* **62,** 3254-3261.

Cirillo, S. L., Lum, J. & Cirillo, J. D. (2000). Identification of novel loci involved in entry by *Legionella pneumophila*. *Microbiology* **146**, 1345-1359.

Clemens, D. L. & Horwitz, M. A. (1992). Membrane sorting during phagocytosis: selective exclusion of major histocompatibility complex molecules but not complement receptor CR3 during conventional and coiling phagocytosis. *J Exp Med* **175**, 1317-1326.

Clemens, D. L. & Horwitz, M. A. (1993). Hypoexpression of major histocompatibility complex molecules on *Legionella pneumophila* phagosomes and phagolysosomes. *Infect Immun* **61**, 2803-2812.

Coers, J., Monahan, C. & Roy, C. R. (1999). Modulation of phagosome biogenesis by *Legionella pneumophila* creates an organelle permissive for intracellular growth. *Nat Cell Biol* **1**, 451-453.

Conover, G. M., Derre, I., Vogel, J. P. & Isberg, R. R. (2003). The *Legionella pneumophila* LidA protein: a translocated substrate of the Dot/Icm system associated with maintenance of bacterial integrity. *Mol Microbiol* **48,** 305-321.

Cossart, P. & Sansonetti, P. J. (2004). Bacterial Invasion: The Paradigms of Enteroinvasive Pathogens. *Science* **304,** 242-248.

Coxon, P. Y., Summersgrill. J. T., Ramirez, J. A., Miller, R. D. (1998). Signal transduction during *Legionella pneumophila* entry into human monocytes. *Infect Immun* **66/6,** 2905-2913.

Cullen, B. R. (2006). Enhancing and confirming the specificity of RNAi experiments. *Nature Methodes* **3 (9),** 677-681.

Delevoye, C., Nilges, M., Dautry-Varsat, A. & Subtil, A. (2004). Conservation of the Biochemical Properties of IncA from *Chlamydia trachomatis* and *Chlamydia caviae* (Oligomerisation of IncA Mediates Interaction between Facing Membranes). *JBC* **279 (45),** 46896–46906.

Derré, I. & Isberg, R. (2005). *Legionella pneumophila* Replication Vacuole Formation Involves Rapid Recruitment of Proteins of the Early Secretory System. *Infect Immun* **72(5),** 3048–3053.

Diao, A., Rahman, D., Pappin, D. J. C., Lucocq, J. & Lowe, M. (2003). The coiled-coil membrane protein Golgin-84 is a novel rab effector required for Golgi apparatus ribbon formation. *JCB* **160 (2),** 201–212.

Dorer, M. S., Kirton, D., Bader J. S., Isberg R. R.. (2006). RNA Interference Analysis of *Legionella* in Drosophila Cells: Exploitation of Early Secretory Apparatus Dynamics. *PLoS Pathogens* **2**, 4 0315-0327.

Dowling, J. N., Saha, A. K. & Glew, R. H. (1992). Virulence factors of the family *Legionellaceae*. *Microbiol Rev* **56**, 32-60.

Dreyfus, L. A. & Iglewsky, B. H. (1986). Purification and characterization of an extracellular protease of *Legionella pneumophila*. *Infect. Immun.* **51**, 736-743.

Duden, R. (2003). ER-to-Golgi apparatus transport: COPI and COPII function. *Mol. Membr. Biol.* **20,** 197–207.

Eckmann, L., Kagnoff, M. F. & Fierer, J. (1993). Epithelial Cells Secrete the Chemokine Interleukin-8 in Response to Bacterial Entry. *Inf & Immun* **61 (11),** 4569-4574.

Edelstein, P. H., Edelstein, M. A., Higa, F. & Falkow, S. (1999). Discovery of virulence genes of *Legionella pneumophila* by using signature tagged mutagenesis in a guinea pig pneumonia model. *Proc Natl Acad Sci U S A* **96**, 8190-8195.

Elbashir, S. M., Martinez, J., Patkaniowska, A., Lendeckel, W. & Tuschl, T. (2001). Functional anatomy of siRNAs for mediating efficient RNAi in *Drosophila melanogaster* embryo lysate. *Embo J.* **20(23),** 6877-6888.

Essig, A., Heinemann, M., Simnacher, U. & Marre, R. (1997). Infection of *Acanthamoeba castellanii* by *Chlamydia pneumoniae. Appl. Environ. Microbiol.* **63 (4)**, 1396-1399.

Fasshauer, D. (2003). Structural insights into the SNARE mechanism. *Biochimica et Biophysica Acta* **1641,** 87–97.

Fasshauer, D., Sutton, R. B., Brunger, A. T., & Jahn, R. (1998). Conserved structural features of the synaptic fusion complex: SNARE proteins reclassified as Q- and R-SNAREs. *PNAS* **95,** 15781–15786.

Fernandez, R. C., Logan, S. M., Lee, S. H. & Hoffman, P. S. (1996). Elevated levels of *Legionella pneumophila* stress protein Hsp60 early in infection of human monocytes and L929 cells correlate with virulence. *Infect Immun* **64**, 1968-1976.

Fettes, P. S., Susa, M., Hacker, J. & Marre, R. (2000). Characterization of the *Legionella pneumophila* gene *lig*A. *Int J Med Microbiol* **290**, 239-250.

Fields, B. S. (1996). The molecular ecology of *Legionellae*. *Trends Microbiol* **4**, 286-290.

Fields, B. S., Benson, R. F. & Besser, R. E. (2002). *Legionella* and Legionnaires' Disease: 25 Years of Investigation. *Clin. Microbiol. Rev.* **15 (3),** 506-526.

Finlay, B. B. & Cossart, P. (1997). Exploitation of mammalian host cell functions by bacterial pathogens. *Science* **276**, 718-725.

Finlay, B. B. & Falkow, S. (1997). Common themes in microbial pathogenicity revisited. *Microbiol Mol Biol Rev* **61**, 136-69.

Flieger, A., Gong, S., Faigle, M., Deeg, M., Bartmann, P. & Neumeister, B. (2000a). Novel phospholipase A activity secreted by *Legionella* species. *J Bacteriol* **182**, 1321-1327.

Flieger, A., Gong, S., Faigle, M., Mayerd, H. A., Kehrerd, U., Mussottere, J., Bartmannf, P. & Neumeister, B. (2000b). Phospholipase A secreted by *Legionella pneumophila* destroys alveolar surfactant phospholipids. *FEMS Microbiol Lett* **188**, 129-133.

Flügel, M. (1999). Molekularbiologische Studien zur Pathogenität und Ökologie von *Legionella pneumophila*. : Dissertation, Universität Würzburg.

Gagnon, E., Duclos, S., Rondeau, C., Chevet, E., Cameron, P. H., Steele-Mortimer, O., Paiement, J., Bergeron, J. J. & Desjardins, M. (2002). Endoplasmic reticulum-mediated phagocytosis is a mechanism of entry into macrophages. *Cell* **110,** 119-131.

Gao, L. Y. & Abu Kwaik, Y. (1999a). Activation of caspase 3 during *Legionella pneumophila*-induced apoptosis. *Infect Immun* **67**, 4886-4894.

Gao, L. Y. & Abu Kwaik, Y. (1999b). Apoptosis in macrophages and alveolar epithelial cells during early stages of infection by *Legionella pneumophila* and its role in cytopathogenicity. *Infect Immun* **67**, 862-70.

Gao, L. Y., Harb, O. S. & Abu Kwaik, Y. (1997). Utilization of similar mechanisms by *Legionella pneumophila* to parasitize two evolutionarily distant host cells, mammalian macrophages and protozoa. *Infect Immun* **65**, 4738-4746.

Gao, L. Y., Harb, O. S. & Kwaik, Y. A. (1998a). Identification of macrophage-specific infectivity loci (mil) of *Legionella pneumophila* that are not required for infectivity of protozoa. *Infect Immun* **66**, 883-892.

Gao, L.-Y. & Abu Kwaik, Y. (2000). The mechanism of killing and exciting the protozoen host *Acanthamoeba polyphaga* by *Legionella pneumophila*. *Environmental Micrbiology* **2**, 79-90.

Gao, L.-Y., Stone, B. J., Brieland, J. K. & Abu Kwaik, Y. (1998). Different fates of *Legionella pneumophila pmi* and *mil* mutants within macrophages and alveolar epithelial cells. *Microbial Pathogenesis* **25,** 291–306.

Garcia-Mata, R., Szul, T., Alvarez, C,, Sztul, E. (2003). ADP-ribosylation factor/ COPI-dependent events at the endoplasmic reticulum-Golgi apparatus interface are regulated by the guanine nucleotide exchange factor GBF1. *Mol Biol Cell* **14**, 2250-2261.

Garduno, R. A., Faulkner, G., Trevors, M. A., Vats, N. & Hoffman, P. S. (1998b). Immunolocalization of Hsp60 in *Legionella pneumophila*. *J Bacteriol* **180**, 505-513.

Garduno, R. A., Garduno, E. & Hoffman, P. S. (1998a). Surface-associated hsp60 chaperonin of *Legionella pneumophila* mediates invasion in a HeLa cell model. *Infect Immun* **66**, 4602-4610.

Ghosh, S. & Karin, M. (2002). Missing Pieces in the NF-κB Puzzle. Rev. *Cell* **109**, 81–96.

Gilmore, T. D., (2006). Introduction to NF-κB: players, pathways, perspectives. *NPG Oncogene* **25**, 6680–6684.

Gleeson, P. A., Lock, J. G., Luke, M. R. & Stow, J. L. (2004). Domains of the TGN: coats, tethers and G proteins. *Traffic* **5,** 315-326.

Grimm, D., Merkert, H., Ludwig, W., Schleifer, K. H., Hacker, J. & Brand, B. C. (1998). Specific detection of *Legionella pneumophila*: construction of a new 16S rRNA-targeted oligonucleotide probe. *Appl Environ Microbiol* **64**, 2686-2690.

Gruenberg, J. & van der Goot, F. G. (2006). Mechanisms of pathogen entry through the endosomal compartments. *Molecular Cell Biol* **7,** 495-504.

Gurkan, C., Lapp, H., Alory, C., Su, A. I., Hogenesch J. B. & Balch W. E. (2005). Large-scale profiling of Rab GTPase trafficking networks: the membrome. *Mol. Biol. Cell* **16,** 3847–3864.

Habyarimana, F., Al-khodor, S., Kalia, A., Graham, J. E., Price, C. T., Garcia, M. T. & Abu Kwaik, Y. (2008). Role for the Ankyrin eukaryotic-like genes of *Legionella pneumophila* in parasitism of protozoan hosts and human macrophages. *Env Microbiol* **10 (6),** 1460–1474.

Häcker, H. & Karin, M. (2006). Regulation and function of IKK and IKK-related kinases. *Sci Sign* **357**

Hacker, J. & Heeseman, J. (2000). Infektionsmodelle. In *Molekulare Infektionsbiologie*. Heidelberg, Berlin: Spektrum Akademischer Verlag.

Hacker, J. (1995). Cloning and genetic characterization of the flagellum subunit gene (*fla*A) of *Legionella pneumophila* serogroup 1. *Infect Immun* **63**, 2499-2507.

Hägele, S., Hacker, J. & Brand, B. C. (1998). *Legionella pneumophila* kills human phagocytes but not protozoan host cells by inducing apoptotic cell death. *FEMS Microbiol Lett* **169**, 51-8.

Hales, L. M. & Shuman, H. A. (1999a). The *Legionella pneumophila rpo*S gene is required for growth within *Acanthamoeba castellanii*. *J Bacteriol* **181**, 4879-4889.

Hales, L. M. & Shuman, H. A. (1999b). *Legionella pneumophila* contains a type II general secretion pathway required for growth in amoebae as well as for secretion of the Msp protease. *Infect Immun* **67**, 3662-3666.

Hammer, B. K. & Swanson, M. S. (1999). Co-ordination of *Legionella pneumophila* virulence with entry into stationary phase by ppGpp. *Mol Microbiol* **33**, 721-731.

Harb, O. S. & Abu Kwaik, Y. (2000). Characterization of a macrophage-specific infectivity locus (*mil*A) of *Legionella pneumophila*. *Infect Immun* **68**, 368-376.

Harb, O. S., Venkataraman, C., Haack, B. J., Gao, L. Y. & Kwaik, Y. A. (1998). Heterogeneity in the attachment and uptake mechanisms of the Legionnaires' disease bacterium, *Legionella pneumophila*, by protozoan hosts. *Appl Environ Microbiol* **64**, 126-132.

Hay, J. C., Chao, D. S., Kuo, C. S. & Scheller, R. H. (1997). Protein interactions regulating vesicle transport between the endoplasmic reticulum and the Golgi apparatus in mammalian cells. *Cell* **89,** 149–158.

Hayden, M. S. & Ghosh, S. (2008). Shared Principles in NF-κB Signaling. *Cell* **132**, 344-362.

Heuer, D., Rejman Lipinski, A., Machuy, N., Karlas, A., Wehrens, A., Siedler, F., Brinkmann, V. & Meyer, T. F. (2009). Chlamydia causes fragmentation of the Golgi compartment to ensure reproduction. *Nature Let.* **457 (5),** 731-735.

Heuner, K. (1994). Herstellung und Screening einer Expressionsgenbank von *Legionella pneumophila* Corby. Diplomarbeit, Universität Würzburg.

Heuner, K., Bender-Beck, L., Brand, B. C., Luck, P. C., Mann, K. H., Marre, R., Ott, M. & Hacker, J. (1997). Molekularbiologische Untersuchungen zur Funktion und Expression der Flagelle von *Legionella pneumophila*. Dissertation, Universität Würzburg.

Heuner, K., Brand, B. C. & Hacker, J. (1999). The expression of the flagellum of *Legionella pneumophila* is modulated by different environmental factors. *FEMS Microbiol Lett* **175**, 69-77.

Heuner, K., Hacker, J. & Brand, B. C. (1997). The alternative sigma factor sigma28 of *Legionell pneumophila* restores flagellation and motility to an *Escherichia coli fli*A mutant. *J Bacteriol* **179**, 17-23.

Hilbi, H., Segal, G. & Shuman, H. A. (2001). Icm/Dot-dependent upregulation of phagocytosis by *Legionella pneumophila. Mol Microbiol* **42**, 603–617.

Hilbi, H. (2006). Modulation of phosphoinositide metabolism by pathogenic bacteria. *Cell Microbiol*, **8,** 1697–1706.

Hoffman, P. S. & Garduno, R. A. (1999). Surface-associated heat shock proteins of *Legionella pneumophila* and Helicobacter pylori: roles in pathogenesis and immunity. *Infect Dis Obstet Gynecol* **7**, 58-63.

Hoffman, P. S., Ripley, M. & Weeratna, R. (1992a). Cloning and nucleotide sequence of a gene (*omp*S) encoding the major outer membrane protein of *Legionella pneumophila*. *J Bacteriol* **174**, 914-20.

Hoffman, P. S., Seyer, J. H. & Butler, C. A. (1992b). Molecular characterization of the 28- and 31- kilodalton subunits of the *Legionella pneumophila* major outer membrane protein. *J Bacteriol* **174**, 908-913.

Hoffmann, A. & Baltimore, D. (2006). Circuitry of nuclear factor □B signaling. *Immunological Reviews* **210**, 171–186.

Hoffmann, A., Levchenko,A., Scott, M. L., Baltimore D. (2002). The IκB–NF-κB Signaling Module: Temporal Control and Selective Gene Activation. Science **298**, 1241-1244.

Holden, D. W. (2002). Trafficking of the *Salmonella* vacuole in macrophages. *Traffic* **3,** 161-169.

Horwitz, M. A. & Maxfield, F. R. (1984). *Legionella pneumophila* inhibits acidification of its phagosome in human monocytes. *J Cell Biol* **99**, 1936-1943.

Horwitz, M. A. (1983). The Legionnaires' disease bacterium (*Legionella pneumophila*) inhibits phagosome-lysosome fusion in human monocytes. *J Exp Med* **158**, 2108-2126.

Horwitz, M. A. (1984). Phagocytosis of the Legionnaires' disease bacterium (*Legionella pneumophila*) occurs by a novel mechanism: engulfment within a pseudopod coil. *Cell* **36**, 27-33.

Horwitz, M. A. (1988). Phagocytosis and intracellular biology of *Legionella pneumophila*. In *NATO ASI Series, Bacteria, Complement and the Phagocytic Cell*. Edited by F. C. Cabello & C. Pruzzo. Heidelberg: Springer Verlag Berlin.

Hryniewicz-Jankowska, A., Czogalla, A., Bok. E. & Sikorsk, A. F. (2002). Ankyrins, multifunctional proteins involved in many cellular pathways. *Folia Histochem Cytobiol* **40,** 239-249.

Hunter, T. (1998). THE CROONIAN LECTURE 1997. The phosphorylation of proteins on tyrosine: its role in cell growth and disease. *Phil.Trans. R. Soc. Lond. B* **353,** 583-605.

Husmann, L. K. & Johnson, W. (1992). Adherence of *Legionella pneumophila* to guinea pig peritoneal macrophages, J774 mouse macrophages, and undifferentiated U937 human monocytes: role of Fc and complement receptors. *Infect Immun* **60**, 5212-5218.

Joshi, A. D., Sturgill-Koszycki, S. & Swanson, M. S. (2001). Evidence that Dot-dependent and -independent factors isolate the *Legionella pneumophila* phagosome from the endocytic network in mouse macrophages. *Cell. Microbiol.* **3,** 99–114.

Kagan, J. C. & Roy, C. R. (2002). *Legionella* phagosomes intercept vesicular traffic from endoplasmic reticulum exit sites. *Nat Cell Biol* **4,** 945-954.

Kagan, J. C., Stein, M. P., Pypaert, M. & Roy, C. R. (2004). *Legionella* subvert the functions of Rab1 and Sec22b to create a replicative organelle. *J Exp Med* **199,** 1201-1211.

Karin, M. & Greten, F. R. (2005). NF-κB: linking inflammation and immunity to cancer development and progression. *Nat. Rev. Immunology* **5**, 749-759.

Karpala, A. J., Doran, T. J. & Bean, A. J. D. (2005). Implications for gene silencing technologies. *Immunol & Cell Biol* **83,** 211–216.

Kearns, J. D., Basak, S., Werner, S. L., Huang, C. S., & Hoffmann, A. (2006). IκBε provides negative feedback to control NF-κB oscillations, signaling dynamics, and inflammatory gene expression. *JCB* **173,** 659–664.

King, C. H., Fields, B. S., Shotts, E. B., Jr. & White, E. H. (1991). Effects of cytochalasin D and methylamine on intracellular growth of *Legionella pneumophila* in amoebae and human monocyte like cells. *Infect Immun* **59**, 758-763.

Kirby, J. E. & Isberg, R. R. (1998). Legionnaires' disease: the pore macrophage and the legion of terror within. *Trends Microbiol* **6**, 256-258.

Kirby, J. E., Vogel, J. P., Andrews, H. L. & Isberg, R. R. (1998). Evidence for pore-forming ability by *Legionella pneumophila*. *Mol Microbiol* **27**, 323-36.

Kishima, H., Takemura, H., Yamamoto, H., Kanemitsu, K., Shimada, J. (2000). Evaluation of the activity of antimicrobial agents against *Legionella pneumophila* multiplying in a human monocytic cell line, THP-1, and an alveolar epithelial cell line, A549. *J Infect Chemother* **6,** 206–210.

Köhler, R,, Fanghänel, J., König, B., Lüneberg, E., Frosch, M., Rahfeld, J. U., Hilgenfeld, R., Fischer, G., Hacker, J. & Steinert, M. (2003). Biochemical and functional analyses of the Mip protein: influence of the N-terminal half and of peptidylprolyl isomerase activity on the virulence of Legionella pneumophila. *Infect Immun. 71 (8),* 4389-4397.

Krappmann, D. & Scheidereit, C. (2005). A pervasive role of ubiquitin conjugation in activation and termination of IκB kinase pathways. *EMBO reports* **6 (4)**, 321-326.

Kubori, T., Hyakutake, A. & Nagai H. (2008). *Legionella* translocates an E3 ubiquitin ligase that has multiple U-boxes with distinct functions. *Molecular Microbiology* **67(6),** 1307–1131.

Laguna, R. K., Creasey, E. A., Li Z., Valtz N. & Isberg R. R. (2006). A *Legionella pneumophila*-translocated substrate that is required for growth within macrophages and protection from host cell death. *PNAS* **103 (49),** 18745–18750.

Lie, D. C., Colamarino, S. A., Song, H. J., Désiré, L., Mira, H., Consiglio, A., Lein, E. S., Jessberger, S., Lansford, H., Dearie, A. R. & Gage, F. H. (2005). Wnt signalling regulates adult hippocampal neurogenesis. *Nature* **437** (7063): 1370–5.

Liles, M. R., Edelstein, P. H. & Cianciotto, N. P. (1999). The prepilin peptidase is required for protein secretion by and the virulence of the intracellular pathogen *Legionella pneumophila*. *Mol Microbiol* **31**, 959-970.

Liles, M. R., Viswanathan, V. K. & Cianciotto, N. P. (1998). Identification and temperature regulation of *Legionella pneumophila* genes involved in type IV pilus biogenesis and type II protein secretion. *Infect Immun* **66**, 1776-1782.

Liu, M., Conover, G. M. & Isberg, R. R. (2008). *Legionella pneumophila* EnhC is required for efficient replication in tumour necrosis factor alpha-stimulated macrophages. *Cell Microbiol.* **10 (9),** 1906-1923.

Losick, V. P. & Isberg, R. R. (2006). NF-κB translocation prevents host cell death after low-dose challenge by *Legionella pneumophila. JEM* **203 (9)**, 2177–2189.

Lowe, M., Lane, J. D., Woodman, P.G. & Allan, V. J. (2004). Caspase-mediated cleavage of syntaxin 5 and giantin accompanies inhibition of secretory traffic during apoptosis. *JCS* **117 (7),** 1139-1150.

Lück & Helbig (1997). *Legionellaceae* (Legionellose). *Blackwell Wissenschafts-Verlag* **46**, 1-12.

Lüneberg, E., Zahringer, U., Knirel, Y. A., Steinmann, D., Hartmann, M., Steinmetz, I., Rohde, M., Kohl, J. & Frosch, M. (1998). Phase-variable expression of lipopolysaccharide contributes to the virulence of *Legionella pneumophila*. *J Exp Med* **188**, 49-60.

Lüneberg, E., Zetzmann, N., Alber, D., Knirel, Y. A., Kooistra, O., Zähringer, U. & Frosch, M. (2000). Cloning and functional characterization of a 30 kb gene locus required for lipopolysaccharide biosysnthesis in *Legionella pneumophila*. *Int. J. Med. Micrbiol.* **290**, 37-49.

Luo, Z. Q. & Isberg, R. R. (2004). Multiple substrates of the *Legionella pneumophila* Dot/Icm system identified by interbacterial protein transfer. *Proc Natl Acad Sci* USA **101,** 841-846.

Machner, M. P. & Isberg, R. R. (2006). Targeting of Host Rab GTPase Function by the Intravacuolar Pathogen *Legionella pneumophila. Developmental Cell* **11,** 47-56.

Machner, M. P. & Isberg, R. R. (2007). A Bifunctional Bacterial Protein Links GDI Displacement to Rab1 Activation. *Science* **318,** 974-977.

Machuy, N., Thiede, B., Rajalingam, K., Dimmler, C., Thieck, O., Meyer, T. F. & Rudel, T. (2005). A Global Approach Combining Proteome Analysis and Phenotypic Screening with RNA Interference Yields Novel Apoptosis Regulators. *Mol & Cell Proteom* **4 (1),** 44-55.

Mancini, M., Machamer, C. E., Roy, S., Nicholson, D. W., Thornberry, N. A., Casciola-Rosen, L. A. & Rosen, A. (2000). Caspase-2 Is Localized at the Golgi Complex and Cleaves Golgin-160 during Apoptosis. *JCB* **149 (3),** 603–612.

Mann, M., Ong, S.-E., Grønborg, M., Steen, H., Jensen, O. N. & Pandey, A. (2002). Analysis of protein phosphorylation using mass spectrometry: deciphering the phosphoproteome. *TRENDS in Biotech* **20,** 261-268.

Marra, A., Blander, S. J., Horwitz, M. A. & Shuman, H. A. (1992). Identification of a *Legionella pneumophila* locus required for intracellular multiplication in human macrophages. *Proc Natl Acad Sci USA* **89**, 9607-9611.

Martens, S., Kozlov, M. & McMahon, H., T. (2007). How Synaptotagmin Promotes Membrane Fusion. *Science* **316.**

Matthews, M. & Roy, C. R. (2000). Identification and subcellular localization of the *Legionella pneumophila* IcmX protein: a factor essential for establishment of a replicative organelle in eukaryotic host cells. *Infect Immun* **68**, 3971-82.

Mattoo, S., Lee, Y. M. & Dixon, J. E. (2007). Interactions of bacterial effector proteins with host proteins. *Current Opinion in Immunology* **19,** 392–401.

Meissner, C. (2006). RNAi-screen nach Faktoren des Golgi Apparates, die die intrazelluläre Vermehrung von *Chlamydia trachomatis* beeinflussen. Diplomarbeit, MPI Berlin

McClain, M. S., Hurley, M. C., Brieland, J. K. & Engleberg, N. C. (1996). The *Legionella pneumophila hel* locus encodes intracellularly induced homologs of heavy-metal ion transporters of Alcaligenes spp. *Infect Immun* **64**, 1532-1540.

McCormack, A. L., **Schieltz, D. M**., **Goode, B**., **Yang, S**., **Barnes & G**., **Drubin, D. (1997).** Direct analysis and identification of proteins in mixtures by LC/MS/MS and database searching at the low-femtomole level. *Anal Chem.* **69 (4),** 767-776.

McDade, J. E., Shepard, C. C., Fraser, D. W., Tsai, T. R., Redus, M. A. & Dowdle, W. R. (1977). Legionnaires' disease: isolation of a bacterium and demonstration of its role in other respiratory disease. *N Engl J Med* **297**, 1197-1203.

Mello, C. C. & Conte, D. (2004). Revealing the world of RNA interference. *Nature* **431,** 338-342.

Miller, E. A., Beilharz, T. H., Malkus, P. N., Lee, M. C., Hamamoto, S., Orci, L. & Schekman, R. (2003). Multiple cargo binding sites on the COPII subunit Sec24p ensure capture of diverse membrane proteins into transport vesicles. *Cell* **114**,497-509.

Molmeret, M. Bitar D. M., Han, L. & Abu Kwaik, Y. (2004). Cell biology of intracellular infection by *Legionella pneumophila*. *Microbes and Infection* **6,** 129-139.

Mossessova, E., Bickford, L. C. & Goldberg, J. (2003). SNARE selectivity of the COPII coat. *Cell* **114**, 483-495.

Moyer, B. D., Allan, B. B. & Balch, W. E. (2001). Rab1 interaction with a GM130 effector complex regulates COPII vesicle *cis*-Golgi apparatus tethering. *Traffic* **2,** 268–276.

Müller, A., Hacker, J. & Brand, B. C. (1996). Evidence for apoptosis of human macrophage-like HL-60 cells by *Legionella pneumophila* infection. *Infect Immun* **64**, 4900-4906.

Murata, T., Delprato, A., Ingmundson, A., Toomre, D. K., Lambright, D. G. & Roy C. R. (2006). The *Legionella pneumophila* effector protein DrrA is a Rab1 guanine nucleotide-exchange factor. *Nature Cell Biology* **8(9)**, 971-989.

Nagai, H., Kagan, J. C., Zhu, X., Kahn, R. A. & Roy, C. R. (2002). A bacterial guanine nucleotide exchange factor activates ARF on *Legionella* phagosomes. *Science* **295,** 679-682.

Nagai, H. & Roy, C. R. (2003). Show me the substrates: modulation of host cell function by type IV secretion systems. *Cell. Mic.* **5 (6),** 373–383.

Nelson, D. E., Ihekwaba, A. E. C., Elliott, M., Johnson, J. R., Gibney, C. A., Foreman, B. E., Nelson, G., See, V., Horton, C. A., Spiller, D. G., Edwards, S. W., McDowell, H. P., Unitt, J. F., Sullivan, E., Grimley, R., Benson, N., Broomhead, D., Kell, D. B., White, M. R. H. (2004). Oscillations in NF-κB Signaling Control the Dynamics of Gene Expression. *Science* **306,** 704-708.

Nichols, B. J., Hugh, R., Pelham, B. (1998). SNAREs and membrane fusion in the Golgi apparatus. Biochimica et Biophysica Acta 1404, 9-31.

Ninio, S. & Roy, C.R. (2007). Effector proteins translocated by *Legionella pneumophila*: strength in numbers. *Trends in Microbiol* **15 (8),** 372-380.

Nuoffer, C., Davidson, H. W., Matteson, J., Meinkoth, J. L. & Balch, W. E. (1994). A GDP-bound form of Rab1 inhibits protein export from the endoplasmic reticulum and transport between Golgi apparatus compartments. *J. Cell Biol.* **125,** 225–237.

Pan, X., Lührmann, A., Satoh, A., Laskowski-Arce, M. A., Roy, C. R. (2008). Ankyrin Repeat Proteins Comprise a Diverse Family of Bacterial Type IV Effectors. *Science* **320,** 1651-1654.

Pawson, T. & Scott, J. D. (1997). Signaling Through Scaffold, Anchoring, and Adaptor Proteins. *Science* **278,** 2075-2080.

Pawson, T. (1994). Tyrosine kinase signalling pathways. *Princess Takamatsu Symp.1994* **24,** 303-322.

Payne, N. R. & Horwitz, M. A. (1987). Phagocytosis of *Legionella pneumophila* is mediated by human monocyte complement receptors. *J Exp Med* **166**, 1377-1389.

Pfeffer, S. & Aivazian D. (2004). Targeting RAB GTPases to distinct Membrane Compartments. *Science* **5,** 886-896.

Pfeffer, S. R. (1999). Transport-vesicle targeting: tethers before SNAREs. *Nat Cell Biol* **1,** E17-E22

Pope, C. D., Dhand, L. & Cianciotto, N. P. (1994). Random mutagenesis of *Legionella pneumophila* with mini-Tn10. *FEMS Microbiol Lett* **124**, 107-111.

Quinn, M. T., Parkos, C. A. & Jesaitis, A. J. (1989). The lateral organization of components of the membrane skeleton and superoxide generation in the plasma membrane of stimulated human neutrophils. *Biochim Biophys Acta* **987**, 83-94.

Rizo, J. & Rosenmund, C. (2008). Synaptic vesicle fusion. *Nat Struct Mol Biol.* **15 (7),** 665–674.

Robinson, C. G. & Roy, C. R. (2006). Attachment and fusion of endoplasmic reticulum with vacuoles containing *Legionella pneumophila*. *Cell. Microbiol.* **8,** 793–805.

Rosenberger, C. M. & Finlay, B. B. (2003). Phagocyte Sabotage: Disruption of Macrophage Signalling by Bacterial Pathogens. *Nat. Rev. Mol. Cell. Miol.* **4,** 385-396.

Rothman, J. E. & Warren, G. (1994). Implications of the SNARE hypothesis for intracellular membrane topology and dynamics. *Current Biology* **4 (3),** 220-233.

Rowbotham, T. (1986). Current views on the relationships between amoebae, *legionellae* and man. *Isr J Med Sci* **22**, 678-689.

Roy, C. R. & Isberg, R. R. (1997). Topology of *Legionella pneumophila* DotA: an inner membrane protein required for replication in macrophages. *Infect Immun* **65**, 571-578.

Roy, C. R., Berger, K. H. & Isberg, R. R. (1998). *Legionella pneumophila* DotA protein is required for early phagosome trafficking decisions that occur within minutes of bacterial uptake. *Mol Microbiol* **28**, 663-74.

Roy, C. R. (1999). Trafficking of the *Legionella pneumophila* Phagosome. *ASM News* **65**, 416-421.

Roy, C. R. & Coers, I. (2000). Exploitation of macrophages as a replication niche by *Legionella pneumophila*: response [comment]. *Trends Microbiol* **8**, 49-50.

Roy, C. R. (2002). Exploitation of the endoplasmic reticulum by bacterial pathogens. *TRENDS in Microbiology* **9,** 418-424.

Saccani, S., Marazzi, I., Beg, A. A., & Natoli, G. (2004). Degradation of Promoter-bound p65/RelA Is Essential for the Prompt Termination of the Nuclear Factor-κB. *Response J. Exp. Med.* **200 (1),** 107–113.

Salcedo, S. P. & Holden, D. W. (2003). SseG, a virulence protein that targets *Salmonella* to the Golgi apparatus network. *EMBO J* **22,** 5003-5014.

Salcedo, S. P. & Holden D. W. (2005). Bacterial interactions with the eukaryotic secretory pathway. *Curr Opin Microbio* **8**, 92–98.

Satoh, A., Wang, Y, Malsam, J., Beard, M. B., & Warren, G. (2003). Golgin-84 is a rab1 Binding Partner Involved in Golgi apparatus Structure. *Traffic* **4 (3),** 153-161.

Scales, S. J., Pepperkok, R. & Kreis T. E. (1997). Visualization of ER-to- Golgi apparatus transport in living cells reveals a sequential mode of action for COPII and COPI. *Cell* **90,** 1137–1148.

Schlessinger J. (2000). Cell Signaling by Receptor Tyrosine Kinases. *Cell* **103,** 211–225.

Schmeck, B., N'Guessan, P.D., Olloman, M., Loren, J., Zahlten, J., Opitz, B., Flieger, A., Suttorp, N. & Hippenstiel, S. (2007). *Legionella pneumophila*-induced NF-κB and MAPK-dependent cytokine release by lung epithelial cells. *Eur Respir J* **29,** 25–33.

Segal, G. & Shuman, H. A. (1998a). How is the intracellular fate of the *Legionella pneumophila* phagosome determined? *Trends Microbiol* **6**, 253-5.

Segal, G. & Shuman, H. A. (1998b). Intracellular multiplication and human macrophage killing by *Legionella pneumophila* are inhibited by conjugal components of IncQ plasmid RSF1010. *Mol Microbiol* **30**, 197-208.

Segal, G., Purcell, M. & Shuman, H. A. (1998). Host cell killing and bacterial conjugation require overlapping sets of genes within a 22-kb region of the *Legionella pneumophila* genome. *Proc Natl Acad Sci U S A* **95**, 1669-1674.

Segal, G. & Shuman, H. A. (1999a). *Legionella pneumophila* utilizes the same genes to multiply within *Acanthamoeba castellanii* and human macrophages. *Infect Immun* **67**, 2117-2124.

Segal, G. & Shuman, H. A. (1999b). Possible origin of the *Legionella pneumophila* virulence genes and their relation to *Coxiella burnetii* [letter]. *Mol Microbiol* **33**, 669-670.

Segal, G., Russo, J. J. & Shuman, H. A. (1999). Relationships between a new type IV secretion system and the *icm/dot* virulence system of *Legionella pneumophila*. *Mol Microbiol* **34**, 799-809.

Segal, G., Feldman, M. & Zusman, T. (2005). The Icm/Dot type-IV secretion systems of *Legionella pneumophila* and *Coxiella burnetii*. *FEMS Microbiol.* **29,** 65–81.

Sexton, J. A. & Vogel, J. P. (2004). Regulation of Hypercompetence in *Legionella pneumophila. Journ of Bacteriol.* **186, (12),** 3814–3825.

Shohdy, N., Efe, J. A., Emr, S. D., Shuman, H. A. (2005). Pathogen effector protein screening in yeast identifies *Legionella* factors that interfere with membrane trafficking. *PNAS* **102(13),** 4866–4871.

Short, B., Haas, A. & Barr, F. A. (2005). Golgins and GTPases, giving identity and structure to the Golgi apparatus. *Biochimica et Biophysica Acta* **1744,** 383– 395.

Shorter, J., Beard, M. B., Seemann, J., Dirac-Svejstrup, A. B., Warren, G., (2002). Sequential tethering of Golgi apparatusns and catalysis of SNAREpin assembly by the vesicle-tethering protein p115. *JCB* **157,** 45-62.

Somsel-Rodman, J. & Wandinger-Ness, A. (2000). Rab GTPases coordinate endocytosis. *J. Cell Sci.* **113,** 183–192.

Söllner, T. H. (2002). Vesicle Tethers Promoting Fusion Machinery Assembly. *Develop Cell Prewiew* **2,** 377–387.

Sönnichsen, B., Lowe, M., Levine, T., Jamsa, E., Dirac-Svejstrup, B., Warren, G. (1998). A role for giantin in docking COPI vesicles to Golgi apparatus membranes. *J. Cell Biol.* **140,** 1013– 1021.

Steinert, M., Emody, L., Amann, R. & Hacker, J. (1997). Resuscitation of viable but nonculturable *Legionella pneumophila* Philadelphia JR32 by *Acanthamoeba castellanii*. *Appl Environ Microbiol* **63**, 2047-53.

Steinert, M., Engelhard, H., Flugel, M., Wintermeyer, E. & Hacker, J. (1995). The Lly protein protects *Legionella pneumophila* from light but does not directly influence its intracellular survival in *Hartmannella vermiformis*. *Appl Environ Microbiol* **61**, 2428-2430.

Steinert, M., Hentschel U. & Hacker J. (2002). *Legionella pneumophila*: an aquatic microbe goes astray. *FEMS Microbiol Rev* 26, 149-162.

Stevenson, M. (2004). Therapeutic potential of RNA interference. *N. Engl. J . Med* **351,** 1772-1777.

Stone, B. J. & Abu Kwaik, Y. (1998). Expression of multiple pili by *Legionella pneumophila*: identification and characterization of a type IV pilin gene and its role in adherence to mammalian and protozoan cells. *Infect Immun* **66**, 1768-1775.

Stone, B. J. & Kwaik, Y. A. (1999). Natural competence for DNA transformation by *Legionella pneumophila* and its association with expression of type IV pili. *J Bacteriol* **181**, 1395-1402.

Sturgill-Koszycki, S. & Swanson, M. S. (2000). *Legionella pneumophila* replication vacuoles mature into acidic, endocytic organelles. *J. Exp. Med.* **9,**1261–1272.

Susa, M., Hacker, J. & Marre, R. (1996). De novo synthesis of *Legionella pneumophila* antigens during intracellular growth in phagocytic cells. *Infect Immun* **64**, 1679-1684.

Susa, M., Hacker, J., Fettes, P. & Marre, R. (1997). *Legionella pneumophila*: Pathogenese und Pathogenität. *Hygiene und Mikrobiologie* **4/97**, 53-57.

Susa, M, Marre, R (1999). *Legionella pneumophila* Invasion of MRC-5 cells induces tyrosine protein phosphorylation *Infect Immun* **67/9**, 4490-4498.

Swanson, M. S. & Isberg, R. R. (1995). Association of *Legionella pneumophila* with the macrophage endoplasmic reticulum. *Infect Immun* **63**, 3609-3620.

Szeto, L. & Shuman, H. A. (1990). The *Legionella pneumophila* major secretory protein, a protease, is not required for intracellular growth or cell killing. *Infect Immun* **58**, 2585-92.

Teng, F., Wang, Y., Tang, B. L. (2001). The syntaxins. *Genome Biology* reviews **2 (11),** 3012.1–3012.7.

Teruya, H., Higa, H., Akamine, M., Ishikawa, C., Okudaira, T., Tomimori, K., Mukaida, N., Tateyama, M., Heuner, K., Fujita, J. & Mori, N. (2007). Mechanisms of *Legionella pneumophila*-induced interleukin-8 expression in human lung epithelial cells. *BMC Microbiology* **7,** 102.

Tilney, L. G., Harb, O. S., Connelly, P. S., Robinson, C. G. & Roy, C. R. (2001). How the parasitic bacterium *Legionella pneumophila* modifies its phagosome and transforms it into rough ER: implications for conversion of plasma membrane to the ER membrane. *J Cell Sci* **114**, 4637-4650.

Tisdale, E. J., Bourne, J. R., Khosravi-Far, R., Der, C. J. & Balch, W. E. (1992). GTP-binding mutants of Rab1 and Rab2 are potent inhibitors of vesicular transport from the endoplasmic reticulum to the Golgi apparatus complex. *J. Cell Biol.* **119,** 749–761.

Venkataraman, C. & Abu Kwaik, Y. (2000). Signal transduction in the protozoan host *Hartmannella vermiformis* upon attachment to *Legionella pneumophila. Microbes & Infection* **2,** 867–875.

Venkataraman, C., Haack, B. J., Bondada, S. & Abu Kwaik, Y. (1997). Identification of a Gal/GalNAc lectin in the protozoan *Hartmannella vermiformis* as a potential receptor for attachment and invasion by the Legionnaires' disease bacterium. *J Exp Med* **186**, 537-547.

Vogel, J. P., Roy, C. & Isberg, R. R. (1996). Use of salt to isolate *Legionella pneumophila* mutants unable to replicate in macrophages. *Ann N Y Acad Sci* **797**, 271-272.

Vogel, J. P., Andrews, H. L., Wong, S. K. & Isberg, R. R. (1998). Conjugative transfer by the virulence system of *Legionella pneumophila*. *Science* **279**, 873-876.

Vogel, J. P. & Isberg, R. R. (1999). Cell biology of *Legionella pneumophila*. *Curr Opin Microbiol* **2**, 30-4.

Weeratna, R., Stamler, D. A., Edelstein, P. H., Ripley, M., Marrie, T., Hoskin, D. & Hoffman, P. S. (1994). Human and guinea pig immune responses to *Legionella pneumophila* protein antigens OmpS and Hsp60. *Infect Immun* **62**, 3454-3462.

Weide, T., Bayer, M., Köster, M., Siebrasse, J-P., Peters, R. & Barnekow, A. (2001). The Golgi apparatus matrix protein GM130: a specific interacting partner of the small GTPase Rab1B. *EMBO* **2 (4),** 336-341.

Weimbs, T., Low, S. H., Chapin, S. J, Mostov, K. E., Bucher, P. & Hofmann, K. (1997). A conserved domain is present in different families of vesicular fusion proteins: A new superfamily *PNAS* **94,** 3046–3051.

Werner, S. L., Barken, D., Hoffmann A. (2005). Stimulus Specificity of Gene Expression Programs Determined by Temporal Control of IKK Activity. *Science* **309**, 1857-1861.

Wiater, L. A., Sadosky, A. B. & Shuman, H. A. (1994). Mutagenesis of *Legionella pneumophila* using Tn903 dlllacZ: identification of a growth-phase-regulated pigmentation gene. *Mol Microbiol* **11**, 641-653.

Wiater, L. A., Dunn, K., Maxfield, F. R. & Shuman, H. A. (1998). Early events in phagosome establishment are required for intracellular survival of *Legionella pneumophila*. *Infect Immun* **66**, 4450-4460.

Wilson, B. S., Nuoffer, C., Meinkoth, J. L., McCaffery, M., Feramisco, J. R., Balch, W. E. & Farquhar, M. G. (1994). A Rab1 mutant affecting guanine nucleotide exchange promotes disassembly of the Golgi apparatus. *J. Cell Biol.* **125,** 557–571.

Wintermeyer, E., Rdest, U., Ludwig, B., Debes, A. & Hacker, J. (1991). Characterization of legiolysin (lly), responsible for haemolytic activity, colour production and fluorescence of *Legionella pneumophila*. *Mol Microbiol* **5**, 1135-1143.

Wintermeyer, E., Flugel, M., Ott, M., Steinert, M., Rdest, U., Mann, K. H. & Hacker, J. (1994). Sequence determination and mutational analysis of the *lly* locus of *Legionella pneumophila*. *Infect Immun* **62**, 1109-1117.

Xu, D., Joglekar, A. P., Williams, J. C. & Hay, J. C. (2000). Subunit structure of a mammalian ER/Golgi apparatus SNARE complex. *JBC* **275,** 39631–39639.

Xu, Y., Martin, S., James D. E., Hong, W. (2002). GS15 Forms a SNARE Complex with Syntaxin 5, GS28, and Ykt6 and Is Implicated in Traffic in the Early Cisternae of the Golgi Apparatus. *MBC* **13,** 3493–3507.

Yaffe, M. B. (2002). Phosphotyrosine-binding Domains in Signal Transduction. *Nature Reviews, MCB* **3,** 177-186.

Yamamoto, Y., Klein, T. W., Shinomiya, H., Nakano, M. & Friedman, H, (1992). Infection of Macrophages with *Legionella pneumophila* Induces Phosphorylation of a 76-Kilodalton Protein. *Infect Immun* 3452-3455.

Yancheng, L. & Luo, Z-Q. (2006). The *Legionella pneumophila* effector SidJ is required for efficient recruitment of endoplasmic reticulum proteins to the bacterial phagosome *Infect Immun* **75 (2),** 592-603.

Zähringer, U., Knirel, Y. A., Lindner, B., Helbig, J. H., Sonesson, A., Marre, R. & Rietschel, E. T. (1995). The lipopolysaccharide of *Legionella pneumophila* serogroup 1 (strain Philadelphia 1): chemical structure and biological significance. *Prog Clin Biol Res* **392**, 113-39.

Zamboni, D. S., McGrath, S., Rabinovitch, M. & Roy, C. R. (2003). *Coxiella burnetii* express type IV secretion system proteins that function similarly to components of the *Legionella pneumophila* Dot/Icm system. *Mol Mic.* **49,** 965-976.

Zamore, P. D., Tuschl, T., Sharp, P. A. & Bartel, D. P. (2000). RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell* **101,** 25-33.

Zerial, M. & McBride, H. (2001). Rab proteins as membrane organizers. *Nat. Rev. Mol. Cell Biol.* **2,** 107–117.

Zhang, T. & Hong, W. (2001). Ykt6 Forms a SNARE Complex with Syntaxin 5, GS28, and Bet1 and Participates in a Late Stage in Endoplasmic Reticulum-Golgi apparatus Transport. *JBC* **276 (29),** 27480–27487.

Zhang, Y., Higashide, W. M., McCormick, B.A., Chen, J. & Zhou, D. (2006). The inflammation-associated *Salmonella* SopA is a HECT-like E3 ubiquitin ligase. *Mol Microbiol* **62(3),** 786–793.

Zou, C. H., Knirel, Y. A., Helbig, J. H., Zahringer, U. & Mintz, C. S. (1999). Molecular cloning and characterization of a locus responsible for O acetylation of the O polysaccharide of *Legionella pneumophila* serogroup 1 lipopolysaccharide. *J Bacteriol* **181**, 4137-4141.

Zuckman, D. M., Hung, J. B. & Roy, C. R. (1999). Pore-forming activity is not sufficient for *Legionella pneumophila* phagosome trafficking and intracellular growth. *Mol Microbiol* **32**, 990-1001.